PEROXIDATIC ACTIVITIES OF TWO SUPEROXIDE SCAVENGERS - CYTOCHROME C AND SUPEROXIDE DISMUTASE

by

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ABSTRACT

Both cytochrome c and superoxide dismutase are superoxide scavengers whose respective physiological functions are well established. Research on these enzymes has undergone intense revival following two discoveries: the role of cytochrome c in apoptosis, and that of superoxide dismutase in familial ALS. In neither case has the cytotoxic action been fully explained in terms of the enzyme's redox properties. We decided therefore to reinvestigate peroxidatic activities of cytochrome c and superoxide dismutase with relevance to positive feedback in apoptosis and ALS.

The data confirm that peroxidatic activity of native Cu,Zn-superoxide dismutase is minimal unless small molecules such as bicarbonate release this activity. Removing zinc increased H2O2-induced oxidation of TMPD by 8-fold compared with the native enzyme, and this enhanced peroxidatic activity was independent of the presence of bicarbonate. The data also confirm the strong peroxidatic activity of cytochrome c. Pre-incubating cytochrome c with H2O2 increased peroxidatic activity about thirty-fold within 10s followed by a sharp decline. The H2O2-induced release in peroxidatic activity is accompanied by aggregation the protein, without detectible scission that might reflect production of "microperoxidase". Taking previous and current data together, cytochrome c clearly has a powerful but latent capability for positive feedback in the oxidative destruction of living cells.

In characterizing the peroxidatic activity of cytochrome c we report: (1) increasing reaction temperature increased peroxidatic activity of cytochrome c, (2) the reaction rate

in HEPES buffer was 30% less than in phosphate buffer, (3) adding EDTA increased reaction rate by 76%, (4) optimal pH for peroxidatic activity was 8, (5) increasing ionic strength slowed the reaction, (5) adding halides increased peroxidatic activity, (3) calcium and zinc inhibited, while copper slowed the reaction, (5) trolox and the flavonoid anthocyanin inhibited by 20% and 85%, respectively; (6) ascorbate inhibited virtually completely until it was all oxidized, after which peroxidatic activity was greater than controls.

Pending further research it seems safe to say that under oxidative stress these novel capabilities for the positive feedback open new avenues for cytotoxic peroxidations. Some of these may well potentiate the apoptotic sequence or participate in the pathological processes of diseases.

DEDICATION

This thesis is dedicated to my father Zhou Wenying (周文英) who died from heart disease two years ago. He had been a constant moral supporter for me in the past when he was alive. His spirituality will continuously encourage me in the future even though he has passed away. He always wanted me to focus only on studying and working without worrying about achievement (只管耕耘，不问收获), but I wish I could achieve one percent of what he had, academically, in my lifetime. I know that I have lost the opportunity to show my appreciation to him in person. This, however, does not and will never change the fact that this thesis is my gift to him.

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I also thank my coworkers in the Bioenergetics Laboratory, Chunmei Li, Bronwen Hide, Julie Chen, and Tony Berendrecht, for their academic help and friendship over the years.
I am truly grateful to my parents for their love and support, and to my relatives in both China, and North American, for their encouragement and help.
Finally, and most importantly, my greatest debt goes to my wife Yu Yao for her ceaseless nagging, and to my daughters Alice for her patience and tolerance, and Olivia for her love and support.

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ABBREVIATIONS

•OH	hydroxyl radical.
AA	atomic absorption spectroscopy.
ATP	adenosine triphosphate.
Cu,Zn-SOD	copper, zinc superoxide dismutase.
Cu-SOD	Zn-depleted superoxide dismutase.
Cyt-c	cytochrome c.
Des	desferrioxamine.
DTPA	diethylenetriaminepentaacetic acid.
EDTA	ethylenediaminetetraacetate.
ELISA	Enzyme-Linked Immunosorbant Assay.
Fe-SOD	iron superoxide dismutase.
GSH	reduced glutathione.
GSSG	oxidized glutathione.
H_2O_2	hydrogen peroxide.
ICP	inductively coupled plasma emission spectrophotometry.
Km	Michaelis constant.
Mn-SOD	manganese superoxide dismutase.
Ni-SOD	nickel superoxide dismutase.
NAD(P)H	reduced form of nicotinamide adenine dinucleotide phosphate.
$O_2^{\bullet-}$	superoxide anion.
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.
TMPD	tetramethyl-1,4-phenylenediamine.
Vmax	maximum velocity.

PREFACE

In addition to the abstract, prefatory pages, and preface, this thesis consists of three sections: a general introduction, experimental studies, and general conclusions. (1) The general introduction section sets the stage for our experimental studies. (2) The experimental studies section describes our studies on the peroxidatic activities of superoxide dismutase and cytochrome c. (3) The general conclusions section deals with some questions arising from our studies and outlines future directions for studies in this area.

The first part of the experimental studies section presents our experimental data regarding conditions under which peroxidatic activity of superoxide dismutase is released. The negative results of synergism between the peroxidatic activities of superoxide dismutase and cytochrome c are also described here. All data cited in this section were obtained by me, using high-resolution data acquisition software.

The second part of the experimental studies section relates to catalysis of the peroxidation of tetramethylphenylenediamine (TMPD) by cytochrome c, which forms the major focus of this thesis. The data show the reactivity of ferricytochrome c as a peroxidatic catalyst. With one exception, all experiments reported in this section were carried out by me. SDS-PAGE studies showing the scission and formation of dimers and trimers of cytochrome c during pre-incubation with H_2O_2 were carried out in collaboration with Yu Yao, then a research assistant with Dr. Amandio Vieira.

GENERAL INTRODUCTION

Overview of goals and experiments

Intracellularly, superoxide dismutase is an antioxidant enzyme catalyzing the disproportionation of the superoxide radical into hydrogen peroxide and molecular oxygen.[1] It plays a fundamental role in the defense against oxygen-derived active species. In addition to its physiological activity, superoxide dismutase displays a peroxidatic action that utilizes its own product hydrogen peroxide as a substrate.[2]

Cytochrome c, too, is an effective scavenger of superoxide. Inhibition by superoxide dismutase on the reduction of cytochrome c followed at 550 nm is widely used as an assay to measure the dismutase activity of superoxide dismutase.[3] The physiological function of cytochrome c is to transfer electrons between cytochrome bc_1 and cytochrome oxidase in the mitochondrial respiratory chain.[4] In addition, cytochrome c has peroxidase activity[5] and is able to catalyze membrane lipid peroxidation by hydrogen peroxide.[6] So far, no functional significance has been assigned to the peroxidatic activity of cytochrome c. Cytochrome c appears to have evolved to serve several different cellular roles, and its biological significance is undergoing a dramatic reappraisal.

In 1993, Rosen and coworkers demonstrated that mutations in the gene for Cu,Zn-SOD are associated with familial amyotrophic lateral sclerosis, a fatal neurodegenerative disease characterized by the selective loss of motor neurons.[7] Since then, more than 90 mutations of Cu,Zn-SOD have been found worldwide in patients with familial

amyotrophic lateral sclerosis;[8] and these mutations have been implicated in the etiology of this disease. Nevertheless, the exact mechanisms underlying neuronal death induced by mutant Cu,Zn-SOD are unknown.

In transgenic mice, the enzyme activity of mutant superoxide dismutase is not significantly diminished.[9,10,11] This finding contradicts the initial hypothesis that lack of enzymatic activity in the mutant Cu,Zn-SODs causes neurodegeneration. Instead, increasing attention has centered on the possible gain or enhancement of a new toxic function.[12] The most plausible harmful non-dismutase function is still considered by many to be enhanced peroxidatic activity of the familial amyotrophic lateral sclerosis-associated mutant Cu,Zn-SOD.[13,14,15]

Conformational change in the mutant Cu,Zn-SOD protein is believed to be the root to this toxic gain-of-function and to the pathogenesis of amyotrophic lateral sclerosis.[16] Zinc affinity of many familial amyotrophic lateral sclerosis-associated mutant SODs is decreased.[17] Zinc plays an important role in stabilizing the structure of Cu,Zn-SOD.[18] The decreased affinity of the enzyme for zinc can lead to the dissociation of zinc from its binding site and change the conformation of mutant Cu,Zn-SOD. Evidently, the Zn-binding site of Zn-deficient superoxide dismutase is very vulnerable to damage by H_2O_2.[19]

What triggered our interest in this study was the possibility of finding a positive feedback mechanism within the chemistry of superoxide dismutase itself. If peroxidatic activity of mutant Cu,Zn-SOD is involved in familial amyotrophic lateral sclerosis, H_2O_2 might

potentially enhance the toxicity of mutant Cu,Zn-SOD by a positive feedback mechanism. The basis to the positive feedback loop would be that a decrease of zinc affinity of mutant Cu,Zn-SOD leads to an irreversible zinc loss due to the damage of zinc binding site by H_2O_2. The resultant irreversible zinc loss is accompanied by a further increase in the mutant protein's activity toward H_2O_2. This positive feedback loop hypothesis fits well with the observation that amyotrophic lateral sclerosis progresses rapidly once the disease is initiated after being latent for several decades. In this model, H_2O_2 enhances the toxicity of mutant Cu,Zn-SOD to motor neurons in amyotrophic lateral sclerosis by damaging the Zn-binding site of the enzyme.

Because the zinc affinity of many familial amyotrophic lateral sclerosis-associated mutant Cu,Zn-SODs is decreased, removing the zinc from the native enzyme might create a modified enzyme reflecting some properties of a familial amyotrophic lateral sclerosis-associated mutant Cu,Zn-SOD.[17,20] For this reason, the objective of first part of the current project was to explore the potentially toxic activities of Zn-depleted Cu,Zn-SOD. We, therefore, examined and measured the effect of incubation of both native and Zn-depleted Cu,Zn-SOD with H_2O_2 on their subsequent peroxidatic activities . Since bicarbonate reportedly mediates peroxidatic oxidation of some substrates by Cu,Zn-SOD,[21] its participation in peroxidatic activities of native and Zn-depleted Cu,Zn-SOD was also studied.

Neural cell death induced by expression of mutant superoxide dismutase bears the hallmarks of apoptosis.[22] Release of cytochrome c is a key event during apoptosis.[23,24,25] However, there is currently no a definitive mechanism for the participation of

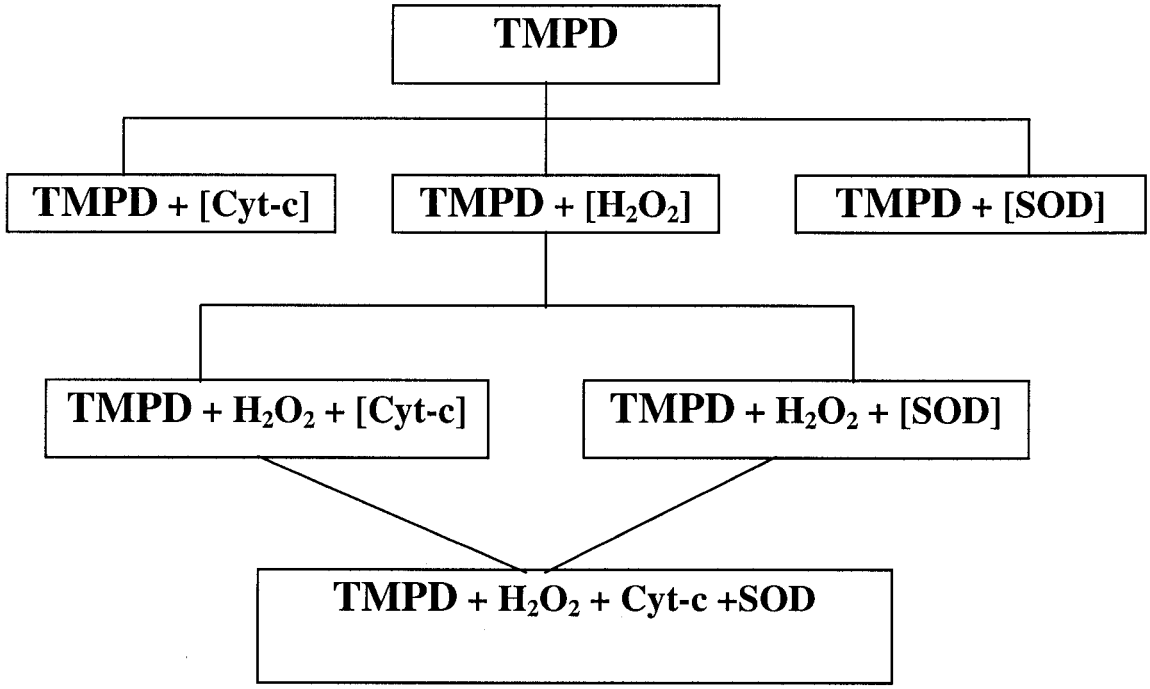
cytochrome c.[26,27] Cells exposed to H_2O_2 undergo apoptosis and H_2O_2 induces the release of cytochrome c from encapsulated artificial mitochondrial membranes.[28,29,30] Based on these observations, one may speculate that H_2O_2 precipitates the release of cytochrome c from mitochondria during apoptosis.

On this basis, I developed the hypothesis that H_2O_2 contributes to the transformation of cytochrome c from a life supporting protein to a life-terminating agent. Specifically, the interaction of cytochrome c with H_2O_2 might change the conformation of cytochrome c to increase its peroxidatic activity. The modified molecules with increased peroxidatic activity might accelerate conversion of other molecules to the enhanced peroxidatic structure, with resultant damage to the outer mitochondrial membrane. This positive feedback might accelerate the release of cytochrome c during apoptosis. The second part of the project, therefore, was to reinvestigate the peroxidatic activity of H_2O_2 treated cytochrome c and the factors affecting this activity. The results of the reinvestigation might provide a clue for such a positive feedback.

Amyotrophic lateral sclerosis is a multifactor disease involving many other factors besides alteration of Cu,Zn-SOD. It seems more useful to look for interactions between the mutant superoxide dismutase and some other cellular regulatory systems as an explanation for the etiology of amyotrophic lateral sclerosis. If apoptosis, and hence cytochrome c are involved in familial amyotrophic lateral sclerosis, it may be rewarding to look for a connection between superoxide dismutase and cytochrome c. To examine the potential synergism between the peroxidatic activities of modified superoxide

dismutase and cytochrome c was also a part of this project, albeit one with almost totally negative results. The indicator of peroxidatic activity used for both cytochrome c and superoxide dismutase was TMPD peroxidation. The following flow chart delineates the experiments I conducted.

Flow Chart



In control studies, I conducted the reactions for (1) TMPD autoxidation, (2) TMPD with superoxide dismutase, and (3) TMPD with cytochrome c. Since the treatment groups examined the peroxidatic reactions of superoxide dismutase and cytochrome c in the presence of hydrogen peroxide, the reaction of TMPD with hydrogen peroxide alone was another necessary control. In the treatment groups, the oxidation of TMPD was studied in the presence of only H_2O_2, and then in the presence of H_2O_2 plus superoxide dismutase or

cytochrome c, first separately, and then together. I tested for a potential synergism between the peroxidatic activities of superoxide dismutase and cytochrome c. In addition, the factors affecting these reactions were also examined. Specifically, I measured the effects of concentration of reagent, buffer species, ionic strength, pH, temperature, incubation time, some cations and anions, metal chelators, hydroxyl radical scavengers, and antioxidants on these reactions.

Structure and activity of Cu,Zn-SOD

Superoxide dismutase was first isolated under the name hemocuprein by Mann and Keilin in 1938,[31,32] and crystallized by Mohamed and Greenberg as erythrocuprein in 1953.[33] The biological enzymatic activity of this protein was discovered by McCord and Fridovich in 1969 when they studied the reduction of cytochrome c by the superoxide radical ($O_2^{\bullet -}$) generated from the xanthine/xanthine oxidase reaction.[3] Currently, various forms of superoxide dismutase are commercially available.

Based on the type of metal contained within the enzyme, superoxide dismutase can be separated into four types: iron superoxide dismutase (Fe-SOD), manganese superoxide dismutase (Mn-SOD), nickel superoxide dismutase (Ni-SOD), and copper, zinc superoxide dismutase (Cu,Zn-SOD).[34] Although these four types of superoxide dismutase have different prosthetic metals, their catalysis of the disproportionation of the superoxide anion are almost the same, since their rate constants are close to diffusion limited, at about $10^9\ M^{-1}\ s^{-1}$ at pH 7.[35] The major difference is that the catalytic rate of the Cu,Zn-SOD is constant from pH 4.5 to 9.5,[36] while those of Fe-SOD and Mn-SOD gradually decrease as the pH increases.[37,38,39,40] The enzymatic activity of Ni-SOD decreases rapidly above pH 9.[41] Due to the current emphasis on Cu,Zn-SOD in relation to familial amyotrophic lateral sclerosis, our emphasis here will be on this type of superoxide dismutase.

Structure of Cu,Zn-SOD

Cu,Zn SOD is a metalloenzyme that contains both copper and zinc atom. Overall, the enzyme is cylindrical, slightly barrel shaped, with its amino sequences well conserved among many species and a high content of basic amino acid residues surrounding its active site.

Cu,Zn-SOD has two identical subunits

Cu, Zn-SOD is a 32 kDa homodimeric antioxidant enzyme made up of two identical subunits, each of which contains 153 amino acids in humans, and 150 amino acids in the bovine form. Each subunit contains one copper and one zinc ion.[42] The copper ion plays an integral role in the disproportionation of the superoxide anion,[3] and the zinc ion is important for the stabilization of the enzyme structure.[43,44] The two subunits associate by noncovalent interactions and the interface is at the front of the β-barrel, slightly left of the center (see "The three-dimensional structure" section for the meaning of β-barrel). The contact between the subunits consists mainly of three polypeptide domains. Among these, two are joined by an intra-chain disulfide bond, and the third is located at the bottom center, bending toward the back of the subunit. The two subunits of the dimer are chemically identical and have identical, or nearly identical, backbone conformations.[45,46,47,48]

Amino acid sequences of Cu, Zn-SOD are highly conserved

The amino acid sequences of Cu, Zn-SOD have been determined in various species. The first third of the initial 40 amino acid residues are invariant over a vast phylogenetic

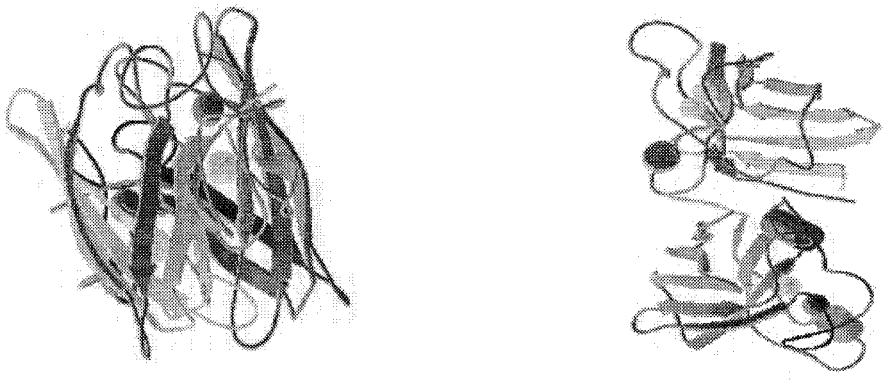
range and at least 50% of the entire amino acid residues are conserved over all forms of Cu,Zn-SOD.[45,49] The six histidine and the one aspartic acid surrounding the metal ligands, the positively charged catalytic site consisting of arginine and lysine, and the cystine disulfide bridge are highly conserved.[50] The sequence conservation of Cu,Zn-SOD is almost as great as that of eukaryotic cytochrome c, implying that the divergence of Cu,Zn-SOD must be very slow.[49] The differences in amino acid sequences between the Cu,Zn-SOD and Fe- or Mn-SOD is as apparent as the similarity within each.[51,52,53,54] The lack of homology between Cu,Zn-SOD, and Fe- or Mn-SOD indicates that they may have converged from two different ancestors, instead of being offspring of divergent evolution from the same ancestor.

The three-dimensional structure of Cu, Zn-SOD reveals its cylindrical barrel shape

The main feature of its tertiary structure is the abundance of ß-sheets in eight antiparallel strands and two loops, which together account for 95% of the entire amino acid sequence. The two loops lie on the surface and tend to extend out from the molecules. They are joined by an inter-chain disulfide bond and contain amino acid residues that are ligands to both the copper and the zinc ions. Each of the loops begins and ends in two adjacent chains on the less regular side of the barrel. One loop (residues 48-79) contains several metal ligands and the half-Cys55 of the disulfide bond, and has two distinct parts. The first part has its end held back against the barrel by the disulfide bridge and participates extensively in the contact between subunits. The second part contributes three ligands to zinc. In contrast, the other loop (residues 119-141) does not have metal ligands nor a

half-cystine, and therefore is not stabilized by metal coordination nor disulfide bonding.[34,48,55]

The interior of the barrel, which is mainly ß-sheet, is packed with hydrophobic side chains. The barrel is slightly flattened in cross section. The portion of barrel toward the outside of the subunits is quite regular, while the portion of the barrel internal to the subunits is less regular and more twisted. It is the ß-barrel structure that makes the enzyme very stable even in extreme conditions.[56,57] The stability of Cu,Zn-SOD is of particular interest because decreased conformational stability of the Cu,Zn-SOD's ß-barrel has been suggested to be a common feature of several familial amyotrophic lateral sclerosis-associated mutant superoxide dismutases.[17,18]



A. Ribbons Structure B. Cylinders structure

Three-dimensional structure of bovine copper-zinc superoxide dismutase[58]

The metal binding sites and active site of Cu, Zn-SOD are crucial for its functions

The copper and zinc ions of Cu, Zn-SOD are located on the "back side" of the molecule, opposite the subunit interface. The two copper ions on the two separate subunits are about 34 Å apart. In each subunit, the copper and zinc are only about 6 Å apart.[48] The copper ion is bound by four histidine residues (His44, 46, 61, and 118). The arrangement and orientation of these histidine ligands make the axial position of the copper much more open on the solvent side than on the protein side, allowing the possibility of binding of a solvent molecule by metal.[59] These four histidine ligands also define a distorted square plane in which the imidazole nitrogen of His61 does not lie in the same plane as the other 3 histidine ligands and the copper, but is located intermediate between planar and axial positions.[60]

The zinc ion is bound by three histidine residues (His61, 69, 78) and one oxygen from the side chain of one aspartic acid residue (Asp81).[61] The zinc ligand geometry is tetrahedral with a strong distortion toward a trigonal pyramid with Asp81 at the apex. The three histidine rings are located slightly in front of the zinc in the direction of the active channel, with Asp81 behind and completely buried.[59]

His61 is a ligand common to both the copper and zinc ions, with the imidazole ring approximately planar to both metals.[50,62] The bridge between copper and zinc breaks when the copper is reduced.[63,64,65,66] Hodgson and Fridovich have postulated that the bridging histidine imidazolate ring may function as a general acid to facilitate protonation of the superoxide anion as it interacts with the reduced enzyme.[67]

As in cytochrome c, the active site of Cu,Zn-SOD consists of a group of positively charged amino acid residues, which create a charged channel with Lys120 and Lys134 at the top and Arg141 inside the channel positioned close (~4-5 Å) to the active site copper.[68] The metals and their seven ligating residues form the floor of the channel with Asp122 buried beneath.[59] This positively charged channel provides an electrostatic attraction to its negatively charged substrate, the superoxide anion, thereby facilitating the association rate by more than 30-fold.[69] These residues are highly conserved during molecular evolution of Cu,Zn-SOD and are crucial to the function of the enzyme. Site-directed mutagenesis of one of these charged amino acids at the active site results in decreased activity,[70] without affecting the metal coordination.[71]

Activity of Cu,Zn-SOD

Cu,Zn-SOD plays an important role in the survival of the organism. It is present in all aerobic organisms, and when human Cu,Zn-SOD genes were inserted into the motor neuron cells of fruit flies, they lived 40% longer.[72] However, as we shall see, there is suggestive evidence for superoxide dismutase toxicity.[73,74]

The biological role of Cu, Zn-SOD is to remove superoxide

Cu, Zn-SOD is an enzyme for defense against oxygen derived active species due to its ability to catalyze the disproportionation of the superoxide anion ($O_2^{\bullet -}$) into O_2 and H_2O_2.[1] Although compared to hydroxyl radicals, superoxide anion is not considered a particularly cytotoxic species, it can interact with and inactivate other antioxidant

enzymes such as catalase[75] and glutathione peroxidase[76]. It also potentially quenches hydroquinone toxicity[77] and reacts with nitric oxide to form peroxynitrite, which is toxic because it can modify cysteine and tyrosine residues on the side chains of proteins.[22,78]

The physiological importance of Cu,Zn-SOD has been indicated by the studies of gene knockout in yeast and transgenic animals. Yeast strains lacking Cu,Zn-SOD are hypersensitive to O_2 and will not grow aerobically.[79] Transgenic strains of the fruit fly, *Drosophila melanogaster*, overexpressing bovine Cu,Zn-SOD were more resistant to hyperoxia than control flies. In contrast, flies lacking Cu,Zn-SOD not only have diminished resistance to the toxicity of O_2 but also have decreased lifespan.[80] Nevertheless, compared with other antioxidant enzymes such as glutathione peroxidase and catalase on a molar basis, Cu,Zn-SOD is less crucial for cell survival against oxidative stress.[81]

Overexpression of Cu,Zn-SOD, however, might not always be beneficial. In Down's syndrome, an extra copy of chromosomes is present due to errors in cell division, resulting in an increase of the Cu,Zn-SOD level by 50% in cells. Reportedly, overexpression of Cu,Zn-SOD sometimes enhances, sometimes decreases, H_2O_2 production.[82] This impairs the transport of neurotransmitters by cultured rat PC12 cells[83] and results in abnormal neuromuscular junctions in the tongue of transgenic mice,[84] apparently similar to those found in tongue muscle of patients with Down's syndrome. Therefore, overexpression of Cu,Zn-SOD might contribute to some of the symptoms observed in Down's syndrome. In reperfused heart, there is an optimal protective dose for superoxide dismutase with deleterious effects at higher dose.[74]

Cu, Zn-SOD catalyzes disproportionation of superoxide anion via an inner-sphere pathway to water and H_2O_2

The superoxide anion can spontaneously dismutate to O_2 and H_2O_2. The uncatalyzed dismutation, however, involves a direct reaction of two superoxide species, and is hindered by electrostatic repulsion between the two superoxide anions.[59] The uncatalyzed dismutation *in vivo* is also affected by the low steady state concentration of superoxide (10^{-10}M).[85] Superoxide dismutase solves these problems by the alternate reduction and oxidation of the enzyme during successive encounters with superoxide anions, thereby catalyzing electron transfer between the two superoxide anions without requiring their direct interaction. Comparison of the rate of the spontaneous dismutation with that catalyzed by superoxide dismutase at physiological pH shows that the enzyme increases the dismutation by 10,000 fold.[59,85,86]

It is believed that during the alternate reduction and oxidation of the enzyme the copper ion first accepts one electron from one superoxide anion yielding O_2 (1), and then donates an electron to a second superoxide anion giving H_2O_2 (2):[3,36,86,87]

$$O_2^{\bullet-} + \text{Cu(II)Zn-SOD} \rightarrow O_2 + \text{Cu(I)Zn-SOD} \qquad (1)$$

$$O_2^{\bullet-} + \text{Cu(I)Zn-SOD} + 2H^+ \rightarrow H_2O_2 + \text{Cu(II)Zn-SOD} \qquad (2)$$

The electron transfer between copper and superoxide can occur through either an inner-sphere or outer-sphere pathway. In the inner-sphere pathway, the electron is transferred through an intermediate in which the reactants share a bridging ligand in their inner coordination shells, while the outer-sphere reaction does not required modification of the

coordination shell of the reactants. The evidence available favors the inner-sphere pathway and supports the mechanism in which the superoxide anion displaces a water molecule from the inner coordination sphere of copper, prior to, or during electron transfer, in both the reduction and oxidation steps of the reaction.[88,89,90,91,92,93]

Arg141 in the active site of the enzyme participates in formation of an intermediate complex between superoxide anion and the enzyme. The analysis of the local environmental effects of Arg141 shows that this residue prevents the reduction of copper by forming a hydrogen bond to superoxide anion and by generating an electric field in the active site that opposes the transfer of an electron from superoxide anion to copper.[94]

The reduction of copper in Cu,Zn-SOD is associated with the breaking of the active site of the bimetal cluster due to the rupture of the His61 imidazolate bridge.[63,64,65,66,95] The breaking of the imidazolate bridge between His61 and copper during the enzymatic catalysis, and consequently the uncoupling of the bimetal cluster leads to the uptake of one proton per subunit by the His61 ring nitrogen not ligated to the zinc.[96,97]

Superoxide anion facilitates the generation of hydroxyl radicals via the Haber-Weiss reaction

Hydroxyl radicals (•OH) are arguably the most reactive chemical species produced in living organisms. They display extremely high rate constants for hydrogen abstraction from a variety of cellular components, including DNA, proteins, membrane phospholipids, carbohydrates, and organic acids.[28,29,30] Hydroxyl radicals are sometimes generated as by-products of normal cellular function. For example, phagocytic cells

produce H_2O_2 and superoxide anion during their oxidative burst. With superoxide anion or some other electron donor as a source of reducing electrons, H_2O_2 can be decomposed to OH^- and •OH radicals through both the Fenton reaction (3) and the Haber-Weiss reaction (4a and 4b),[31] now better known as the superoxide anion driven Fenton reaction.

$$H_2O_2 + M^{n+} \rightarrow M^{(n+1)+} + OH^- + \bullet OH \quad (3)$$

$$M^{n+} + O_2^{\bullet -} \rightarrow M^{(n-1)+} + O_2 \quad (4a)$$

$$M^{(n-1)+} + H_2O_2 \rightarrow M^{n+} + OH^- + \bullet OH \quad (4b)$$

Intracellularly, any residual H_2O_2 from the above reactions is subsequently removed by other antioxidant enzymes such as glutathione peroxidase and catalase.[98,99]

Although catalase has a higher turnover number, glutathione peroxidase has a higher affinity for H_2O_2 than catalase,[81] and it is therefore more effective at the lower H_2O_2 concentrations (5):

$$2GSH + H_2O_2 \xrightarrow{\text{glutathione peroxidase}} GSSG + 2H_2O \quad (5)$$

The oxidized glutathione (GSSG) can then be re-reduced to GSH at the expense of nicotinamide adenine dinucleotide phosphate primarily from the pentose phosphate pathway.

The contribution of catalase in the degradation of H_2O_2 increases as H_2O_2 concentration increases.[100,101] Catalase accelerates the following overall reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (6)$$

Cu,Zn-SOD and familial amyotrophic lateral sclerosis

Although amyotrophic lateral sclerosis has been known for more than hundred years, its cause is still poorly understood. The discovery of a linkage between mutations of the Cu,Zn-SOD gene and familial amyotrophic lateral sclerosis provides a starting point. Initially it was hypothesized that the mutations resulted in a lack of superoxide dismutase activity, which in turn caused familial amyotrophic lateral sclerosis. However, this concept was discarded in light of the equivalence of Cu,Zn-SOD activity in mutant and native forms. The prevailing view now is that the mutant Cu,Zn-SOD has acquired some new function that renders it toxic. This concept is referred to as the "gain-of-function" hypothesis. One possibility is that the mutant Cu,Zn-SOD has acquired some pro-oxidant activity. Emerging evidence suggests that a conformational change in the mutant Cu,Zn-SOD is central to this toxic "gain-of-function" and to the pathogenesis of familial amyotrophic lateral sclerosis. If this is the case, the importance of the zinc and its binding site of the mutant enzyme and its impact on the pro-oxidant activity of Cu,Zn-SOD needs to be addressed because zinc plays a crucial role in stabilizing the structure of the enzyme.

Amyotrophic lateral sclerosis is a fatal adult-onset neurodegenerative disease

The average age of onset of amyotrophic lateral sclerosis is approximately 50 years, with a prevalence of ~5 per 100,000 individuals.[102,103] This human disease, first described by Charcot more than 130 years ago,[104] is characterized by the selective degeneration of

motor neurons, and manifests itself as a progressive decline in muscular function due to skeletal muscle atrophy. Consequently, such a degeneration results in eventual paralysis, speech deficits, and ultimately death due to respiratory failure, usually within 2 to 5 years. At present, this disease is untreatable and its etiology is still unknown.

Cu,Zn-SOD mutations are the only known etiological clue for amyotrophic lateral sclerosis

The most important clue we have to the mechanism of amyotrophic lateral sclerosis comes from the discovery by Rosen and coworkers of mutant Cu,Zn-SOD in some patients with familial amyotrophic lateral sclerosis.[7] This finding prompted tremendous interest among scientists in the fields of biomedical research. To date, more than 90 mutations have been found worldwide in 20% of patients with familial amyotrophic lateral sclerosis.[8,105]

The gene of Cu,Zn-SOD is located on the long arm of chromosome 21, and includes five exons and four introns with a length of about 11 kb. The mutations observed currently involve 40 of the 153 amino acid residues, located in all five exons. All of these mutations are autosomal dominant, with one exception. This exception involves the substitution of alanine for aspartate at position 90 (D90A). This mutation can be either recessive (in Scandinavians and their descendents),[106,107] or dominant (in non-Scandinavian populations).[108,109] Among the 90+ familial amyotrophic lateral sclerosis-associated mutations, A4V is the most common Cu,Zn-SOD mutation, and it is often found in patients with the most rapid progression, and particular short duration of

disease.[110,111] G37R, G41D, G93C, and D100G are frequently associated with relatively long duration of disease, while L38V, G37S are usually associated with early onset.[105,112]

The mutations identified in the gene of Cu,Zn-SOD in patients with familial amyotrophic lateral sclerosis, however, could initially be considered simply as polymorphisms on the human genome across the normal population. By definition, a locus is polymorphic if there are two or more alleles, each of which has a frequency of 1% or more.[113] For example, in the genes of ABO blood group system of human populations, allele I^A varies in frequency between 20% and 30%, allele I^B varies between 10% and 20%, and allele i, which produce no antigen, varies between 60% and 70%. Thus, the ABO blood group system is a polymorphic trait.[114] The polymorphism sometimes can be identified by restriction fragment length polymorphism (RFLP) analysis. The changes of base in polymorphism might create new, or abolish old, sites for restriction enzymes. Digestion with these enzymes then produces DNA fragments of varying length, which can be separated by gel electrophoresis. DNA microarray, a new technology that is reshaping molecular biology, is a powerful tool that will undoubtedly shed further light on the polymorphism as researchers apply the technique to the problems of amyotrophic lateral sclerosis.

The frequency of Cu,Zn-SOD mutations is much lower than 1% in human population. Many people with amyotrophic lateral sclerosis do not have the mutation, and many people with the mutation do not have amyotrophic lateral sclerosis. Nevertheless, it was concluded that these mutations are not simple polymorphisms, but disease related. This conclusion is not based on all the mutations identified in the Cu,Zn-SOD gene in patients

with familial amyotrophic lateral sclerosis but on the calculation of odds ratio. The odds ratio indicates the chances of suffering some fate, and can be computed using the following formula:

$$\text{Odds ratio} = \{p1/(1-p1)\}/\{p2/(1-p2)\}$$

In the current example, p1 and p2 represent the fraction of familial amyotrophic lateral sclerosis patients among people who have Cu,Zn-SOD mutations and the fraction of familial amyotrophic lateral sclerosis patients among people who do not have Cu,Zn-SOD mutations, respectively. If the odds ratio is large, the probability is high that the mutations are disease related. For example, if the odds ratio is 19, probability that the mutations of Cu,Zn-SOD are disease related is 95% {19/(19+1)}. If the odds ratio is around 1, it means that there is a no difference between two groups (p1 and p2) and the mutations of Cu,Zn-SOD gene represent merely a simple polymorphic variability.

Rosen and coworker examined two out of five exons of the Cu,Zn-SOD gene in DNA samples from patients with familial amyotrophic lateral sclerosis and control normal individuals. They identified 7 variants of exon 2, 11 variants of exon 4 in 148 and 150 familial amyotrophic lateral sclerosis patients, respectively. However, no variant was detected in the control groups (140 and 112, respectively, for exons 2 and 4).[7] If we put these values into the above formula, the apparent odds ratio is infinite because of the zero value for variants in the control group.

Since familial amyotrophic lateral sclerosis accounts for about only 5-10% of all cases of amyotrophic lateral sclerosis and the prevalence of amyotrophic lateral sclerosis is about 5 per 100,000 individuals,[102,103] the number of familial amyotrophic lateral sclerosis patients in the general population is proximately 2.5-5 per 1,000,000. Conservatively, if we take this value as the percentage of familial amyotrophic lateral sclerosis patients among people who do not have Cu,Zn-SOD mutations, we have an odds ratio of 12857-25714. The ratio of the odds for and against the particular degree of linkage is known as the logarithm of the odds, or lod score. If the lod score is 3 or higher, it usually indicates a significant likelihood of linkage. On the other hand, if the lod score is 2 or lower, it usually indicates a significant probability of no linkage. Based on our conservative estimation for odds ratio of familial amyotrophic lateral sclerosis, we have a lod score of 4.1-4.4 for the mutation of Cu,Zn-SOD in familial amyotrophic lateral sclerosis. These data, therefore, indicate that the mutations of Cu,Zn-SOD are disease related.

The nature of the toxicity of the mutated Cu,Zn-SOD in amyotrophic lateral sclerosis is still unknown

Although Rosen and coworkers' discovery is an important development in research on amyotrophic lateral sclerosis, the linkage of mutations of Cu,Zn-SOD with familial amyotrophic lateral sclerosis, however, does not mean that the mutations cause familial amyotrophic lateral sclerosis. That is, familial amyotrophic lateral sclerosis might merely co-exist with mutations of the gene. To prove that the mutations in the Cu,Zn-SOD gene cause familial amyotrophic lateral sclerosis, we have to examine the consequence of the mutations and whether they influence the pathological process. Mutations of a gene

usually result in an alteration of the quality and quantity of its protein products. If the protein product is an enzyme, the resultant disease is caused either by a loss-of-function or by a gain-of-function of this enzyme. These changes of enzyme function can be characterized by biochemical analysis. Since Cu,Zn-SOD is an enzyme, the mutations of its gene might lead to the loss of its enzyme activity, or the gain of a new function. Therefore, to prove the etiological role of mutations in the Cu,Zn-SOD gene in familial amyotrophic lateral sclerosis, we need to investigate whether the mutations cause a loss-of-function or a gain-of-function.

In vivo, the loss of enzyme activity should cause accumulation of superoxide anion because the physiological activity of Cu,Zn-SOD is removal of superoxide anion. Initially, the loss-of-function hypothesis made sense because the accumulation of reactive oxygen species might lead to the damage of motor neuron cells and thus the onset of familial amyotrophic lateral sclerosis.

To test the loss-of-function hypothesis site-directed mutagenesis has been extensively used to create samples of familial amyotrophic lateral sclerosis-associated mutant Cu,Zn-SOD. These are then examined for altered enzymic activities. Researchers have also measured the enzyme activity of mutant Cu,Zn-SOD from patients with familial amyotrophic lateral sclerosis to see whether the enzyme activity is decreased in parallel with the severity of the disease. In a more direct approach, researchers have used animal models. These studies, however, did not show any correlation between Cu,Zn-SOD activity and the frequency or severity of disease in patients with familial amyotrophic

lateral sclerosis as well as transgenic mice in which the Cu,Zn-SOD gene has been deleted.[9,11] More importantly, mutant Cu,Zn-SOD transgenic mice developed motor neuron loss in spite of having normal endogenous superoxide dismutase activity.[9,12] In addition, mice with the Cu,Zn-SOD gene deleted did not develop a spontaneous motor neuron disease.[10] These findings, therefore, refuted the loss-of-function hypothesis and led to a wide spread acceptance that an increase in some non-dismutase activity of the mutant enzyme is the cause of amyotrophic lateral sclerosis. Such a mechanism would, moreover, be consistent with the autosomal dominant pattern of inheritance.

The cytotoxic 'gain-of-function' hypothesis gets further support from the observation that expression of familial amyotrophic lateral sclerosis mutant human Cu,Zn-SOD is pro-apoptotic in cultured neuronal cells, while wild-type human Cu,Zn-SOD is anti-apoptotic.[115] The gain-of-function explanation will, however, remain a hypothesis until its nature is identified. Furthermore, failure to show one potential new function of mutant enzyme cannot rule out the possibility of other unknown functions that might cause the disease. Finding a new function of mutant enzyme also does not mean that this new function is the cause of disease. Looking for the potential toxic new function, again, may best be done by directly measuring the potential toxic activities of mutant Cu,Zn-SODs from patients with familial amyotrophic lateral sclerosis or from the enzymes expressed from genetically manipulated genes. We can also explore the potential toxic activity by using chemically modified Cu,Zn-SODs, such as the Zn-depleted superoxide dismutase used in the current studies.

Because of the widespread belief that some 'gain-of-function' is associated with the mutant Cu,Zn-SODs found in familial amyotrophic lateral sclerosis, the nature of this 'gain-of-function' is being actively sought. One plausible candidate for the 'gain-of-function' of Cu,Zn-SOD is the mutant enzyme's peroxidatic activity.[13,14,15,116] The peroxidatic activity leads to generation of hydroxyl type radicals, among others, capable of damaging cellular targets including DNA, protein, and lipid membranes. A4V, G93A, and H48Q mutants reportedly have enhanced peroxidatic activity compared to the wild-type enzyme, particularly at lower H_2O_2 concentrations.[14,15,117] The simplest explanation for the enhanced peroxidatic activity of mutant enzymes is that the mutant proteins are more accessible to H_2O_2 due to a more accessible active center.[58] Evidence for this argument includes elevated hydroxyl radical generation in mice expressing mutant Cu,Zn-SOD,[118] and oxidative damage in neuronal tissue in amyotrophic lateral sclerosis.[119]

It is generally assumed that increase of accessibility to H_2O_2 also increases the susceptibility of Cu,Zn-SOD to peroxidatic inactivation. This is because H_2O_2 readily reduces the enzyme-bound Cu^{2+} to Cu^{1+} and generates •OH by a site-specific Fenton reaction involving the Cu^{1+}-bound histidine and another H_2O_2, causing the inactivation of the enzyme.[67,120,121]

Familial amyotrophic lateral sclerosis-associated mutant Cu,Zn-SODs, such as E100G, G93A, and G93R, can be inactivated by H_2O_2.[122] One can further speculate that a positive feedback in the peroxidatic activity of the familial amyotrophic lateral sclerosis-associated Cu,Zn-SOD variants might play a role in the development of the disease.

Currently, however, no solid experimental evidence is available to support this hypothesis.

Decrease of affinity of the mutated Cu,Zn-SOD for zinc may increase its toxicity in amyotrophic lateral sclerosis

Many mutations in Cu,Zn-SOD associated with amyotrophic lateral sclerosis alter the Zn-binding site.[17,20] The mutated enzyme's affinity for zinc is decreased up to 30-fold relative to the wild type.[17] Zinc plays an important role in stabilizing the structure of the enzyme by helping the two loops come together to form a narrow active site around the copper.[18] Improper zinc binding might therefore contribute to the toxic gain-of-function in mutant Cu,Zn-SOD. Conversely, the decrease in affinity for zinc in mutant Cu,Zn-SOD could cause dissociation of the zinc from its binding site, resulting in formation of active species as acceleration of the reverse reaction converting oxygen to superoxide anion.[123]

The ability of Cu,Zn-SOD to catalyze the reaction of nitric oxide and superoxide anion to form peroxynitrite is another candidate for the enzyme's 'gain-of-function'.[124] Peroxynitrite is a strong oxidizing and nitrating agent that can react with many classes of biomolecules. For example, tyrosine residues in proteins can be nitrosylated by peroxynitrite. Oxidative stress mediated by nitric oxide and its toxic metabolite peroxynitrite has reportedly been associated with amyotrophic lateral sclerosis.[125] Superoxide anion produced through reversal of the superoxide dismutase reaction can combine with freely diffusing nitric oxide, producing peroxynitrite. The decrease in zinc

affinity can induce a subtle alteration in protein conformation so that peroxynitrite, instead of its normal substrate, superoxide, can access the active center.[22] The Zn-depleted protein reportedly converts peroxynitrite more readily into nitronium ions than does the Zn-containing enzyme.[21] Support for this concept is provided by the increased level of free 3-nitrotyrosine in the spinal cords of human amyotrophic lateral sclerosis patients and in mouse models of amyotrophic lateral sclerosis.[119,126,127,128]

In contrast, however, there is no evidence of increased protein-bound nitrotyrosine in amyotrophic lateral sclerosis patients or in transgenic mice.[129] Failing to detect nitrated peptide or nitrotyrosine on any target protein challenges the relevance of nitrotyrosine to amyotrophic lateral sclerosis *in vivo*.[130] In addition, neuronally derived nitric oxide reportedly plays no role in the pathogenesis of amyotrophic lateral sclerosis.[131] Therefore, the significance and origin of the 'free' nitrotyrosines remains unclear.

Cu,Zn-SOD can react with its own product H_2O_2

The peroxidatic ability of Cu,Zn-SOD to use its normal product, H_2O_2, was first reported by Hodgson and Fridovich.[67,132] This reaction is favored only when the pH is raised above 8, since the pK_a for H_2O_2 is 11.9.[133] Recently, Sankarapandi and Zweier have documented that bicarbonate is required for Cu, Zn-SOD to display its peroxidatic activity at physiological pH.[21]

The reaction of Cu,Zn-SOD with H_2O_2 slowly and irreversibly inactivates the enzyme,[67] evidently because of the oxidation of the active site histidine, His118.[67,134,135,136,137]

According to Hodgson and Fridovich, H_2O_2 first reduces the Cu^{2+} and then reacts with the Cu^{1+} to give a Cu^{2+}-bound •OH, which can attack an adjacent histidine and destroy the integrity of the catalytic site.[67] Yim and coworkers, however, argued against this mechanism and suggest that the •OH, instead of being restricted to the active site of the enzyme, is released from the active site.[138,139] In contrast, Sankarapandi and Zweier have provided evidence against the generation of free •OH.[140]

Another suggestion is that H_2O_2 damages the copper-binding domains, resulting in the release of free copper. This copper can catalyze free •OH generation via Fenton-type reactions.[141] Despite claims that the peroxidatic functions of Cu,Zn-SOD is a 'gain-of-function' of mutant Cu,Zn-SOD associated with familial amyotrophic lateral sclerosis,[13,14,15,116] such claims to date lack convincing support.

Familial amyotrophic lateral sclerosis accounts for about only 5-10% of all cases of amyotrophic lateral sclerosis. The remaining 90-95% of amyotrophic lateral sclerosis cases are sporadic; with no clue regarding their etiology. Nevertheless, the sporadic and familial forms of amyotrophic lateral sclerosis are clinically indistinguishable, raising the possibility that they share a common etiology. These observations support the contention that in the sporadic form of the disease an altered superoxide dismutase might trigger the onset, perhaps because of a "post-translational modification" of the enzyme.

Such a post-translational change to the native Cu,Zn-SOD would presumably give it characteristics similar to the familial amyotrophic lateral sclerosis-associated mutant

enzymes. The need for this change to spread throughout the population of Cu,Zn-SOD molecules requires a positive feedback mechanism. One possibility is that H_2O_2 damage to the active site (copper-bound) histidine makes the copper even more accessible to H_2O_2, giving the damaged product significant peroxidatic activity. In this speculation the damaged enzyme can peroxidatically target other Cu,Zn-SOD molecules. As they suffer similar active site damage, they, in turn, gain enhanced peroxidatic activity.

In both the sporadic and familial forms of the disease, models for the etiology are constrained by the need to explain (1) why the onset of the disease occurs so often at age around 50, and (2) why progress is so rapid once the disease is initiated. Clearly the mutant Cu,Zn-SOD in the familial form of amyotrophic lateral sclerosis does not cause any observable damage for several decades, yet, once triggered, the process is so aggressive that deterioration and death are usually swift and relentless.

Structure and activity of eukaryotic cytochrome c

Cytochrome c is a general name covering a variety of species-specific c-type cytochromes. The small size, stability, ease of extraction and purification, and commercial availability have made this globular protein a favored target for research in electron transfer reactions. In addition, the heme moiety allows changes in reduction status and certain changes in the structure of the protein to be easily monitored spectrophotometrically.[142] Therefore, cytochrome c has become one of the most studied proteins.

A structural characteristic feature of cytochromes c is that they have one or several porphyrin heme prosthetic groups covalently attached via one or two, thio-ether bridges between cysteine residues of the protein and the vinyl side chains of the heme. Four porphyrin nitrogens are attached to the heme iron. The fifth heme iron ligand is a histidine residue. According to their sequences and three-dimensional structures, cytochromes c are classified into four distantly related types.[143]

Functionally, cytochrome c is a double agent for life and death in living cells. It assists with production of life-sustaining ATP by acting as an electron carrier in the mitochondrial respiratory chain between cytochrome bc_1 and cytochrome oxidase. If it is released from mitochondria, it also triggers apoptosis by which the cell dies through a preprogrammed process. The central role of cytochrome c in apoptosis has rekindled interest in this protein. Here I will review features of its structure and reactivity that are potentially relevant to its newly defined roles.

Determining what changes cytochrome c from a life supporter to a deadly killer is an important area of current research. Cells exposed to H_2O_2 undergo apoptosis,[28,29] confirming that oxidative stress alone can cause apoptosis. A candidate mechanism is available in the finding that H_2O_2 peroxidatically induces the release of cytochrome c from liposomes, a mimic of the mitochondrial membrane.[30] This raises the question of whether H_2O_2 alone causes lyses of the liposomes, or if cytochrome c cooperates in its own release. Our research will address its reactions with H_2O_2, the peroxidase activity of intermediates and products, and their potential role in apoptosis. Our goal is to learn more about the factors that determine whether cytochrome c acts for the benefit or the

detriment of the cell. Opportunities of a positive feedback in this process will provide perspective for the experimental studies of the peroxidatic activities of cytochrome c.

Structure of eukaryotic cytochromes c

The biological functions of cytochromes c are rooted in their structure. Although the structures of cytochrome c from many species have been determined, I will consider here the most extensively characterized type of cytochromes c ---the eukaryotic cytochromes c.

All eukaryotic cytochromes c have 103-113 amino acid residues with a high content of basic amino acid residues

Eukaryotic cytochromes c are encoded by a nuclear gene and synthesized as a cytoplasmic precursor molecule, apocytochrome c, which becomes selectively imported into the mitochondrial inter-membrane space where a heme group is attached by heme lyase.[144,145,146,147] Cytochromes c are small, having only 103-113 amino acid residues. The highly conserved main chain structures and the heme crevice among eukaryotic cytochromes c result from the highly conserved amino acid sequences.[142,148] These conserved residues also determine post-translational modification (folding, and coupling to the heme group), and electron transfer. Among about 100 species of eukaryotic cytochromes c, 23 of these residues are identical and only 14 of the remaining residues are highly variable.[148,149]

All eukaryotic cytochromes c have a high content of basic amino acid residues resulting in isoelectric points (p*I*) greater than 10. Except for two buried arginines and a few lysine

residues, most of the base residues are around the heme crevice. The preservation of lysine residues surrounding the exposed heme edge is crucial.[142] This array of positive charges plays an important role in the recognition and binding of the anionic substrates to cytochrome bc_1 and cytochrome oxidase. For instance, horse heart cytochrome c contains 19 lysine residues; the lysine residues with greatest effect on the reaction rates or binding constants are at positions 13, 86, 87, and 27 at the site for binding cytochrome bc_1, and 13, 72, 86, 87 and 8, the site for binding cytochrome oxidase.[150]

A heme prosthetic group is the main structural feature of eukaryotic cytochromes c

The primary structural feature of eukaryotic cytochromes c is a heme prosthetic group at the active site covalently bound to two cysteine residues (Cys14 and Cys17) through two thio-ether bridges. The axial ligands to the heme are provided by the sulfur atom of Met80 and one of the nitrogen atoms of His18-imidazole. The covalent attachment of heme to protein is one of the most important steps of post-translational modification. This attachment is catalyzed by heme lyase, and coupled to the translocation of cytochrome c across the mitochondrial outer membrane.[151,152] Binding of the heme via thio-ester bonds to Cys14 and Cys17 stabilizes the heme against separating, even under denaturing conditions.[142] The heme group is almost completely buried within the protein matrix, with only a few heme atoms exposed to the surrounding solvent.

The eukaryotic cytochrome c molecule has the dimensions 25 x 25 x 37 Å. Highly refined tertiary structures are currently available for five separate eukaryotic cytochromes c. These are: two isozymes of yeast cytochrome c (iso-1-reduced state at

1.2 Å resolution[153] and iso-1-oxidized state at 1.9 Å resolution,[154] iso-2-reduced state at 1.9 Å resolution[155]); tuna cytochrome c (reduced state at 1.5 Å resolution[156] and oxidized state at 1.8 Å resolution[157]); horse heart cytochrome c (oxidized state at 1.9 Å resolution[158]); and rice cytochrome c (oxidized state at 1.5 Å resolution[159]). All of these cytochromes c share a common polypeptide fold, with the significant conformational differences occurring mainly in flexible surface loops.[158]

The three-dimensional structure of cytochrome c forms a shell around the heme

Since the oxidized form of horse heart cytochrome c was the first cytochrome c to be subjected to X-ray structural analyses,[160] its three-dimensional structure is considered here as an example.[158] Horse heart cytochrome c consists of 104 amino acid residues in a single polypeptide chain with molecular weight 12.4 kDa.[161] The polypeptide chain has six β–turns and five α-helices (three major and two minor), with about 45% of the amino acid residues involved and folded into a roughly globular shape.[158] The average main-chain torsion angles for all residues having an α-helical conformation are in agreement with those found in other high-resolution structures of cytochromes c.[162,163] In addition to the helical elements, residues 37 to 40 and 57 to 59 form a short two-stranded anti-parallel β-sheet, which contains only three inter-strand main-chain hydrogen bonds.[158]

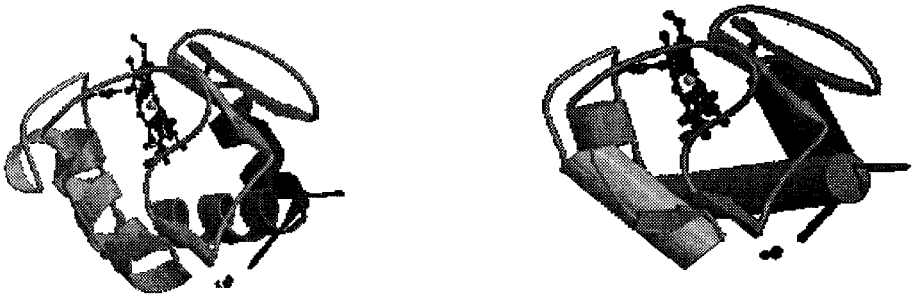
The polypeptide chain forms a shell around the heme, leaving only one edge exposed. The residues around the heme group are bulky and hydrophobic, and the majority of exposed surface residues are charged. The charged residues are distributed around the surface in an asymmetrical manner with the positively charged residues around the

exposed heme area.[164] This distribution is believed to be important to interactions with other electron transfer proteins.[142]

Around the exposed heme is a region of positively charged residues

The heme group of eukaryotic cytochrome c is saddle-shaped. Four atoms of the heme group are exposed to solvent and all of these four atoms are positioned on the front edge of the heme. Consequently, only 7.5% of the total heme surface can directly interact with external solvent molecules.[165]

Heme iron coordinate bond distances are typical of small-molecule six-coordinate iron-porphyrin complexes.[166] The out-of-plane coordinate bond involving the His18 ligand shows only a slight deviation from perpendicular to the pyrrole nitrogen atom and porphyrin ring planes. A larger deviation is observed in the case of the Met80 ligand out-of-plane coordinate bond. The coordination of the iron to the heme requires the dissociation of the pyrrole hydrogen atoms and therefore the porphyrin of the heme has two negative charges. The heme iron atom is not significantly displaced from the two defined heme planes towards either axial ligand. The imidazole ring of His18 lies approximately perpendicular (76°) to the pyrrole nitrogen atom plane of the heme.[158]



A. Ribbons' Structure B. Cylinders' Structure

Three-dimensional structure of horse heart cytochrome c[158]

The amount of structural water in cytochrome c depends on its species and oxidation status

In addition to covalently binding to the side chains of cysteines, heme propionate groups also form a number of hydrogen bonds with nearby polar main-chain and side-chain groups, and structural water that is buried in the heme crevice.[158] Amino acids forming heme propionate interactions are highly conserved in the primary sequences of eukaryotic cytochromes c.[167]

The number of structural water molecules encapsulated inside cytochrome c differs in different species. In horse heart cytochrome c, there are six structural water molecules in the reduced form and five in the oxidized form, with residence times not much greater than a few hundred picos.[168] In tuna cytochrome c, twelve water molecules are found in identical positions in both oxidized and reduced forms.[157]

Certain buried water molecules such as Wat166 and Wat121 are conserved among all eukaryotic cytochromes c.[155,158,168] Wat166 is located at the left side of the heme crevice and forms three hydrogen bonds with the side-chain of the highly conserved residues Asn52, Tyr67, and Thr78. It is involved in both oxidation states, and pH-dependent, conformation changes of the molecule.[157] The buried structural water molecules in the heme crevice contribute to the solvent reorganization energy associated with electron transfer.[168]

The oxidation state of cytochrome c determines its conformation

While the overall structure of cytochrome c is similar in the oxidized and reduced forms, there are significant oxidation state-dependent conformational changes common to various species.[154,169,170,171,172,173,174] Overall, the reduced form is more compact and stable, less compressible, and less viscous in solution; it is more resistant to proteolytic digestion, ligand displacement by exogenous substituents, and to denaturation induced chemically or thermally.[175,176] The increased stability of the reduced form of cytochrome c is due in part to the 1000-fold increase in strength of the Met80-heme iron coordinate bond.[166]

The major conformational differences between the two oxidation states are heme distortion, the orientation of the axial histidine ligand, the formation and breaking of a few hydrogen bonds involving heme propionate-7, and variations in some water molecules surrounding the heme.[171] For example, in tuna cytochrome c, there are substantial conformational changes in the bottom half and on the Met80 side of the heme,

especially in the vicinity of Wat166 which forms hydrogen bonds to the conserved residues Asn52, Tyr67, and Thr78.[156,157,177] After the cytochrome c is oxidized, Wat166, which is buried at the lower left of the heme crevice, shifts 1 Å toward the heme iron. Simultaneously, the Tyr67 swings back and closer to the ligand guided by a hydrogen bond to Wat166. Asn52, which also is hydrogen bonded to Wat166, is pulled upward with it. This causes the nearby Tyr48 side group to move upward and toward the front of the molecule.

The counter-motion of the other oxygen atom of the buried carboxyl group toward the back of the molecule pushes Tyr59 away from the heme by 0.4 Å. Slight upward movement of Tyr48 and Thr49, as well as the upward motion of Tyr67 and Asn52, push the heme up and outward by 0.16 Å. This shift also affects the movements of the adjacent residues Cys17, His18, Trp59, Phe82, and Ala83. The hydrogen bond between Met80 and Tyr67 is broken after oxidation. The shift of Wat166 and a slight outward movement of the heme give the heme a more hydrophilic environment in the oxidized form of cytochrome c.[157]

The differences in structure between oxidized and reduced forms hindered X-ray crystallography of reduced cytochrome c. Attempts to produce crystals of reduced cytochrome c by diffusing hydrogen into crystals of oxidized cytochrome c were frustrated when the crystals shattered because of the conformational change.

pH also affects conformation changes in cytochrome c

Spectroscopically, there are five forms of ferricytochrome c (the oxidized form of cytochrome c) and three forms of ferrocytochrome c (the reduced form of cytochrome c) over the pH range 0 to 13.[177] Form III of ferricytochrome c and form II of ferrocytochrome c are the native cytochromes c and they are stable between pH 4 and pH 8. Around pH 9, form III ferricytochrome c is converted to its alkaline form IV. The conversion involves a slight conformational rearrangement of the molecule resulting from deprotonation of one or more groups, followed by the displacement of the Met80 heme ligand by some other strong-field ligand. The conversion also results in a decrease of reduction potential, making the protein unable to be reduced by cytochrome c reductase.[178,179] The loss of Met80 ligation in alkaline isomerization results in a disappearance of the absorbance band at 695 nm, which can be easily monitored spectrophotometrically. Since the conversion inactivates the cytochrome c and the conversion can occur under physiological conditions, it might play a role in regulating the activity of the electron transport chain.[180]

The alkaline transition of ferrocytochrome c, however, does not occur until pH 12.[181] The greater resistance of the reduced form may be due to the relative lack of positive charges on the heme iron to attract hydroxide ions to the heme edge, and the greater distance (5.4 Å) between the Wat166 and Met80. These two groups might weaken the S-Fe(II) bond and keep the sixth ligand intact even after the hydrogen bond between Wat166 and Thr78 is broken.[182]

As the pH decreases, the acid transitions of ferricytochrome c and ferrocytochrome c occur at pH 2.5 and 4, respectively. A different internal water molecule may be involved in the acid transition. This water is half buried and is only 4.4Å away from His18 so it can pass a proton from the outside medium to His18. The peptide chain is unfolded after His18 is protonated because the protonated imidazole cannot function as an iron ligand.[182]

The heme and its axial ligands dictate the absorption spectra of cytochrome c

The UV-visible spectra of cytochrome c are mainly due to the electronic structure of the heme and its axial ligands, and the majority of the observed bands are due to the electronic transitions in the porphyrin orbitals. Reduced cytochrome c has three intense absorbance bands that are located 550-558 nm (α), 520-527 nm (β), and 415-423 nm (γ). The γ band, the most intense one, is also called the Sorêt band. In oxidized cytochrome c, the α and β band merge to produce a broad band centered at 528 nm and the γ band decreases in intensity and shifts to 409.5 nm. Oxidized cytochrome c also has a weak conformation-dependent absorbance band at 695 nm due to the electronic transition from porphyrin to iron.[148]

The iron atom of the heme of cytochrome c is relatively inert. It does not readily undergo autoxidation at physiological conditions and resists binding by exogenous ligands, such as cyanide and carbon monoxide. When the iron is oxidized, the coordination of the sulfur atom of Met80 to the heme iron is associated, resulting in an absorbance at 695 nm.[183] This band is often used as an index of structural integrity.

Activity of eukaryotic cytochromes c

Cytochrome c is found in all aerobic (oxygen-using) cells, from yeast to multicellular animals. Its vital functions include assisting with production of ATP and triggering apoptosis when it is released from mitochondria.

The biological role of cytochrome c is to relay electrons in the mitochondrial electron-transport chain

The biological role of cytochrome c is to relay electrons from the second proton-pumping complex (cytochrome bc_1) to the third proton-pumping complex (cytochrome oxidase). Cytochrome c transports electrons through a reversible cycling between the 2+ and 3+ oxidation states of the active-site heme iron. The driving force for electron transfer to or from cytochrome c comes from its reduction potential, which falls in the midpoint of reduction potentials of cytochrome bc_1 and cytochrome oxidase. The exchanges of this electron transfer allow its chemiosmotic coupling to ATP formation, which requires that ferrocytochrome c not leak electrons by reacting directly with oxygen. In contrast, as discussed later, ferrocytochrome c readily reduces H_2O_2.

The reduction potential of cytochrome c is affected by many factors

Nearly all eukaryotic cytochromes c have a reduction potential between 260 mV and 290 mV,[184] and the reduction potentials are generally constant over a range of pH from 5 to 8.[185] The reduction potential of cytochrome c is crucial to the function of the protein because it determines its ability to react with its preceding and succeeding reduction partners in the electron transport chain. The reduction potential of cytochrome c is

determined by three independent structural factors: the peripheral side chains of the heme pyrrole rings, the axial ligands to the heme iron, and the local environment of the heme moiety.[148,186,187,188]

The effect of the peripheral side chains of the heme pyrrole rings is inductive. These electron-withdrawing groups increase the positive charge at the heme iron, increasing the reduction potential. Accordingly, saturation of the vinyl group of the protoheme in cytochrome c after forming the thio-ether bridges reportedly decreases the reduction potential of the heme iron. This is inferred because the reduction potential of the heme iron of Fe-mesoporphyrin is 40 mV lower than that of Fe-protoporphyrin, in which the ethyl groups are replaced by vinyl groups.[189]

The influence of the heme ligands on the reduction potential depends on the electron-donating ability of the ligating groups. By donating electrons, the ligating groups partially neutralize the positive charge of the heme iron and therefore decrease the reduction potential.[190] For example, replacing the weak electron donating residue Met80 with a more strongly electron donating His residue results in a decrease of reduction potential.[191] Clearly, the Met80 ligating to the heme iron plays an important role by contributing to the high positive reduction potential of cytochrome c.

The local environment of the heme includes the polarity of the heme microenvironment, the degree of solvent accessibility to the heme, and electrostatic interactions between the heme and neighboring charged groups. An increase in solvent accessibility to the heme increases the polarity of the heme microenvironment. Thus, the first two factors are

actually, to some extent, related to each other. Since all eukaryotic cytochromes c have the same porphyrin and axial ligands, the differences in reduction potential must come primarily from the local environment of the heme moiety.

Heme complexes have a more positive reduction potential in non-polar solvents than the corresponding heme complex in aqueous solution.[192] Thus, by comparing a series of heme proteins of known structure, Stellwagen has noted that the percentage of surface of the heme exposed to the aqueous solvent correlates negatively with the high reduction potential.[193] That the heme group of cytochrome c is buried in a hydrophobic pocket contributes to the high reduction potential of the protein. The small exposed surface of the heme also increases the stability of the reduced state of the heme by restricting its accessibility to exogenous oxidants.

As mentioned previously, there is a high content of basic residues around the exposed edge of the heme group. In addition to facilitating the interaction with other electron donor proteins, these positively charged residues also contribute to the high reduction potential of heme iron.

Cytochrome c accepts or donates electrons, by changing the valence state of the heme iron

Cytochrome c is a reversible, single electron carrier of the mitochondrial inter-membrane space and its main function is to transfer electrons from cytochrome bc_1 to cytochrome c oxidase without significant loss of free energy. The soluble nature of cytochrome c gives it the ability to diffuse readily between cytochrome bc_1 and cytochrome oxidase

complexes. By changing the valence state of the heme iron atom between 2+ and 3+, cytochrome c donates and accepts electrons and acts as an electron carrier in the respiratory chain.[184]

$$\text{Cytochrome c[Fe(III)]} + e^- \Leftrightarrow \text{Cytochrome c[Fe(II)]}$$

Oxidized Reduced

In addition to this primary function, cytochrome c is also involved in several other electron transfer pathways. For example, in animals, cytochrome c acts as an electron acceptor from both cytochrome b_5[194,195] and sulfite oxidase.[196] In yeast, cytochrome c accepts electrons from flavocytochrome b_2 (which catalyzes the oxidation of L-lactate to pyruvate)[197,198] and donates electrons to cytochrome c peroxidase.[199]

Cytochrome c binds tightly to its reduction partners, with a dissociation constant in the range 1-5 x 10^{-7} M. The rate constants of electron transfer reactions are second-order kinetics. The values of these rate constants depend on both the association constant for inter-molecular complexation, and the first order rate constant for electron transfer within its precursor complex. The rate constants for electron transfer reactions between cytochrome c and cytochrome c oxidase, cytochrome bc_1, cytochrome b_5, and cytochrome b_2 are 1.0 x 10^7 $M^{-1}s^{-1}$, 1.7 x 10^7 $M^{-1}s^{-1}$, 4.0 x 10^7 $M^{-1}s^{-1}$, and 2.0 x 10^7 $M^{-1}s^{-1}$, respectively.[184] These cytochrome c-mediated electron transfer reactions occur at rates close to the diffusion limit.[184,200]

Cytochrome bc_1 complex, sometimes called ubiquinol-cytochrome c reductase, is the second of three proton pumps in the respiratory chain. It catalyzes the transfer of

electrons from ubiquinol to cytochrome c, and concomitantly pumps protons across the inner mitochondrial membrane. Ubiquinol transfers one of its two high-potential electrons to the iron-sulfur cluster in the cytochrome bc_1 complex. This electron is in turn transferred to the heme of cytochrome c_1 and cytochrome c, which then carries the electron to the third and the last proton pump, the cytochrome c oxidase complex. The transfer of the electron results in the metamorphosis of ubiquinol into a semiquinone.

Cytochrome c oxidase catalyzes the translocation of electrons from reduced cytochrome c to molecular oxygen, the final electron acceptor in the respiratory chain. Four electrons are funneled into molecular oxygen to completely reduce it to water and concomitantly pump protons from the matrix to the cytosolic side of the inner mitochondrial membrane.[4]

Electron transfer in cytochrome c is accomplished via an outer-sphere pathway

As mentioned before, the intermolecular electron transfer reactions involving co-ordination complexes can occur by either an inner-sphere or outer-sphere pathway. In contrast to the electron transfers carried out by superoxide dismutase, the majority of electron transfers carried out by cytochrome c are via an outer-sphere pathway.[201,202,203,204,205] The outer-sphere electron transfer pathway is based on the activated complex theory (i.e. the reaction proceeds via an intermediate transition state of maximum free energy).[206] The rate constant (k) of an outer sphere reaction can be written as:

$$k = \kappa Z^{-\Delta G/RT}$$

In this equation, κ is the probability of electron transfer occurring when the precursor complex is at the transition state and its value is dependent on the distance of the electron transfer sites, their relative orientation, and the nature of the intervening medium. Z is the collision frequency that has different values in different types of reactions. For example, in bimolecular reactions it is 10^{11} $M^{-1}s^{-1}$, while in unimolecular reactions it is 10^{13} $M^{-1}s^{-1}$. ΔG is the free energy difference between the ground state and transition state within the precursor complex. R is the gas constant, which in an ideal gas equals 8.314 J/mol. T is the temperature in degrees Kelvin.

Electron transfer by cytochrome c can be explained by the Marcus theory

Many theories have been proposed to interpret mechanisms of the electron transfer reactions, including those carried out by cytochrome c. Among these is the "Marcus theory", for which R. A. Marcus was awarded the Nobel Prize in chemistry in 1992. According to this theory,[207] the electron transfer reaction is not like the reactions involving a transfer of atoms or groups between reactants that require a rearrangement of atoms between the reactants. Such rearrangements of atoms require a large spatial overlap of the electronic orbitals of the reacting molecules in the transition state. Therefore, there is a strong interaction of the electronic structures of the two reactants in the transition state in these reactions.

In contrast, electron transfer reactions do not need to break chemical bonds nor rearrange atoms; they involve only the transfer of an electron between the reacting molecules. For such reactions, only a slight overlap of the electronic orbitals is required to overcome the

energy barrier to electron transfer. Therefore, there is only a slight electronic interaction involved in electron transfer reactions. Based on the Marcus theory the generalized electron transfer reaction can be written:

$$D + A \Leftrightarrow X^* \Leftrightarrow X \Leftrightarrow D^+ + A^-$$

In this equation, D and A stand for the reactants, and D^+ and A^- stand for products. Both X and X* indicate transition states with the same atomic configuration (the sum of the atomic configuration of the reacting pair and the solvent) but different electronic configuration. X* has an electronic configuration which is the same as that of the reactants. X* can alter the reactants either by disorganization of some of the oriented solvent molecules, or by spontaneously changing to state X via electronic reorganization. X has the same electronic configuration as that of the product, and this state can either revert to X* by an electronic transition, or move apart to form the products.

Peroxidatic activity of cytochrome c

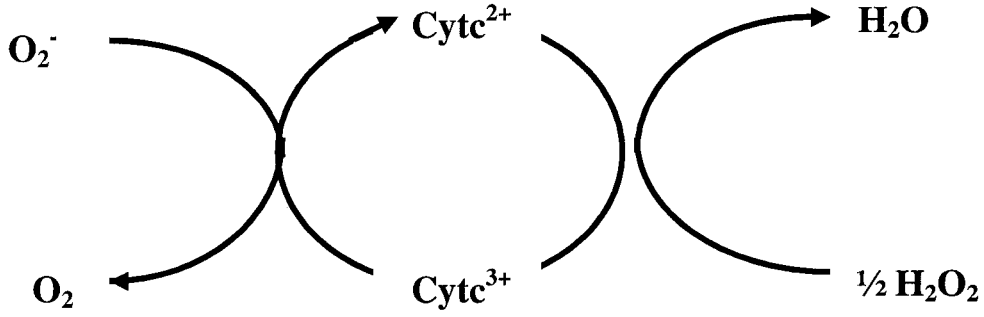
Peroxidatic activity of cytochrome c implies the ability of this protein to react with H_2O_2 and this reaction is autocatalytic. Furthermore, the cleavage of cytochrome c by protease increases its peroxidatic activity if the resultant fragment still contains the prosthetic heme group.

Cytochrome c has significant reactivity toward H_2O_2 and $O_2^{\bullet-}$

According to the reduction potential of cytochrome c, the oxidation of ferrocytochrome c by oxygen is thermodynamically favorable at neutral pH. This reaction, however, is very slow and only becomes fast in the presence of salt.[208] In contrast, the oxidation of ferrocytochrome c by H_2O_2 is fast. Its rate constant observed in our laboratory is 5.51 x

10^6 $M^{-1}s^{-1}$at pH 8.1, 21°C.[209] When cytochrome c becomes oxidized, it can catalyze the oxidation of various electron donors in the presence of H_2O_2, including 2-2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), 4-aminoantipyrine, and luminol.[210] This peroxidatic activity of cytochrome c in the presence of H_2O_2 generates strong oxidants that can attack polyunsaturated fatty acid and cause lipid peroxidation. Ferricytochrome c can catalyze the H_2O_2 induced peroxidation of phosphatidylcholine liposomes[6] as well as the lipid peroxidation of rat heart mitochondria.[211]

It does not appear to have been noted in the literature that the combined superoxide scavenger and peroxidatic activity allow cytochrome c a unique and different enzymatic activity. Cytochrome c, however, potentially has a secondary role as a superoxide dismutase (O_2^-:H_2O_2 oxidoreductase).



Cytochrome c fuctions as a superoxide dismutase

It is not a new finding that cytochrome c has peroxidatic activity. Cytochrome c shares a common prosthetic heme group with horseradish peroxidase and catalase whose primary functions are to catalyze the reaction of H_2O_2. The prosthetic heme group plays a key role for the peroxidatic activities of these proteins.

Peroxidation of ferrocytochrome c is autocatalytic

The peroxidation of ferrocytochrome c is autocatalytic, i.e., the reaction can be catalyzed by its own product ferricytochrome c. The rate of oxidation of ferrocytochrome c by H_2O_2 is initially slow and this slow initial rate is followed by acceleration due to catalysis by the product.[212] The inhibition of peroxidation by cyanide and azide (two ligands that can form complexes with ferricytochrome c) also reflects autocatalysis. Comparison of oxidation of ferrocytochrome c at 550 nm with loss of its Sorêt peak at 409 nm indicates that major degradation of cytochrome c does not begin until all the ferrocytochrome c has been oxidized.[212] Therefore, the catalysis of peroxidation of ferrocytochrome is by the native protein instead of its degraded form.

"Microperoxidase" – cytochrome c fragments have enhanced peroxidase activity

Surprisingly, certain proteolytic cytochrome c fragments containing the heme group ("microperoxidase") have stronger peroxidase activity than the intact cytochrome c. For example, cytochrome c fragment 11-21 has a 20-fold greater peroxidatic activity than intact horse heart cytochrome c at pH 4.9.[213] Fragment 14-21, derived from fragment 11-21 via removing three residues with trypsin, also has an enhanced peroxidatic activity.[214] The peroxidatic activity of horse heart cytochrome c fragment 1-65 is 88 and 875 times that of the whole protein at pH 3.0 and at pH 7.0, respectively.[215,216] Thus, a major factor in the evolutionary design of the holoenzyme may be a minimization of its peroxidatic activity. As a corollary, small conformational changes may dramatically increase the peroxidatic activity of cytochrome c.

Cytochrome c and apoptosis

The linkage of cytochrome c and apoptosis rests on the observation that release of cytochrome c from mitochondria into the cytosol triggers apoptosis. However, none of the currently proposed hypotheses can satisfactorily explain the process of cytochrome c release from mitochondria during apoptosis.

Peroxidatic activity of cytochrome c may mediate apoptosis

In the previous section, I mentioned that cytochrome c fragments have an enhanced peroxidatic activity. At present, no functional significance has been assigned to the enhanced peroxidatic activity induced by scission. However, there is fuel for speculation in that peroxidatic processes mediate apoptosis. The implication is that once a doomed cell invokes the peroxidatic phases of apoptosis, cytochrome c can act as a switch that, by a positive feedback, dramatically amplifies cytotoxic peroxidatic processes. Thus, the peroxidatic activity of cytochrome c might contribute to the trigger for apoptosis.

Apoptosis has specific morphological characteristics

Apoptosis is a preprogrammed process by which cells die in response to environmental or developmental cues. Morphologically, this programmed cell death or cell suicide is characterized by cytoplasmic bleb formation, chromatin condensation, and nucleosomal fragmentation.[217] Specifically, when apoptosis occurs, the apoptotic cell becomes loose or detached from its neighbours and shrinks. The cellular junctions and microvilli on the plasma membrane disappear,[218] and the cell membrane becomes contorted and folded into blebs. The cytoplasm buds and forms apoptotic bodies containing cell organelles.[219]

Phagocytic cells engulf these apoptotic bodies, and their contents are degraded after fusing with lysosomes. The chromatin is condensed near the nuclear membrane. The cleavage between nucleosomes leads to a rapid degradation of nuclear DNA, resulting in nucleosomal fragmentation of 180 to 200 base pairs, which give a typical "ladder" pattern on an electrophoretic gel.

Defects in the apoptotic machinery cause many diseases

Apoptosis is a ubiquitous process in multi-cellular organisms to eliminate unwanted or damaged cells.[220] It provides an organism with a safe and efficient way to continuously turnover cells in any tissue, and the capability to remove specific cells during development. It is crucial for the development and maintenance of tissue homeostasis in multi-cellular organisms.[221] By means of apoptosis, the organism destroys cells, including those mutated or infected with viruses, which represent a threat to the integrity of the organism.

Defects in the apoptotic machinery are associated with many diseases. For example, cells respond to DNA damage by increasing their production of p53, a tumor suppressor gene product that can trigger apoptosis. If the normal apoptosis of these damaged cells becomes inappropriately deactivated, these cells may mutate and become cancer cells.[222] Potentially autoreactive lymphocytes during development, and excess effector cells when immune responses wane, are removed by apoptosis to prevent attack on body constituents. The deactivation of apoptosis in these potentially autoreactive lymphocytes and excess effector cells may result in autoimmune disease.[223] The consequences of faulty apoptosis necessitate that once initiated it is a multifaceted chain reaction involving

a positive feedback. On the other hand, if apoptosis is inappropriately activated in cells necessary for normal functioning, such as those in the nervous system, the consequence may be neurodegenerative disorders like amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease.

Release of cytochrome c from mitochondria triggers apoptosis

A key event in apoptosis is the release of cytochrome c from mitochondria to cytosol.[23] Microinjecting cytochrome c into various cell types also triggers apoptosis.[24,25] Two mechanisms reportedly mediate cell killing by cytochrome c released from the mitochondria.[26,27] One involves activation of the caspase (a cysteine protease that cleave after aspartic acid residues) cascade by interaction of the released cytochrome c with apoptosis activating factor 1 (Apaf-1) and pro-caspase-9 in the cytosol.[224] The other one depends on retardation of mitochondrial electron transport subsequent to cytochrome c release, which in turn impairs ATP production and promotes the generation of reactive oxygen species.[225] A pronounced change in cytochrome c that precedes overt signs of apoptosis is the exposure of an otherwise hidden epitope.[226] Display of a new cytochrome c epitope is highly specific for pre-apoptotic cells, and does not occur in cells destined to survive. Thus, the conformation change of cytochrome c plays a role during the apoptotic processes.

Mechanism of cytochrome c release during apoptosis is still not clear

Although cytochrome c release is a typical theme in many experimental models of apoptosis, the mechanism of its translocation across the outer mitochondrial membrane is unclear. Two primary hypotheses attempt to explain the relocation of cytochrome c during apoptosis.

The first hypothesis attributes the release of cytochrome c to rupture of the outer mitochondrial membrane, a result of swelling of the mitochondrial matrix due to the opening of a large conductance channel.[227,228,229] This channel is a permeability-transition pore composed of several polypeptides,[227,230,231,232] and is believed to comprise the adenine-nucleotide translocator and the voltage-dependent anion channel. According to the hypothesis, the opening of this nonselective channel by permeability-transition pore openers (including Bax or Bid) via binding to adenine-nucleotide translocator,[233] increase the permeability of the inner membrane, resulting in mitochondrial depolarization.[234] The consequence of the above events is the entry of water and solutes into the mitochondria, causing mitochondrial matrix swelling. Inhibiting the permeability-transition pore pharmacologically protects the cell from dying in several models of apoptosis.[235] This hypothesis, however, cannot explain why in certain conditions there is either no loss of membrane potential or the loss of membrane potential occurs after the release cytochrome c from mitochondria during apoptosis.[236,237,238,239]

The second hypothesis suggests that the release of cytochrome c from mitochondria is due to the formation of a pore in the mitochondrial membrane that is large enough to

allow the passage of cytochrome c and other mitochondrial proteins into the cytosol. Potential candidates for the formation of this pore are two of the pro-apoptotic members of the Bcl-2 family: Bax and Bid.[240,241,242]

The oligomer of Bax can form a pH-sensitive, voltage-dependent ion-conducting channel in artificial lipid bilayers and Bcl-2 inhibits this formation.[243,244,245] Bax reportedly cooperates with the voltage-dependent anion channel to form a cytochrome c-conducting channel.[246] The normal voltage-dependent anion channel is not large enough to allow cytochrome c to pass through. When binding Bax, voltage-dependent anion channel changes its conformation, leading to the formation of a larger channel that is permeable to cytochrome c. The release of cytochrome c from mitochondria induced by Bax does not involve mitochondrial swelling,[240] and is insensitive to permeability-transition pore inhibitor.[244] Bax can make the membrane less stable by decreasing the linear tension of phospholipid bilayers,[247] and facilitate its insertion and oligomerization within membranes, leading to formation of hydrophobic pores.

Bid reportedly mediates the mitochondrial damage and release of cytochrome c induced by caspase 8 in response to activation of cell surface death receptors such as Fas and tumor necrosis factor.[241,242] Bid changes the conformation of Bax after it translocates to mitochondria and binds to Bax, and consequently causes the release of cytochrome c from mitochondria.[248] Bid, therefore, relays an apoptotic signal from the cell surface to mitochondria.

In contrast, the anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-x_L block the release of cytochrome c into the cytosol of intact cells.[238,239,249, 250,251,252,253,254] Bcl-2 or Bcl-x_L may form small conductance channels in mitochondrial membranes to counterbalance the channel formation effect of Bax by maintaining electrical homeostasis and membrane integrity.[255,256] The channel properties of Bcl-2 might be regulated by Raf-1 kinase and CED-4 homologs.[253,257,258,259,260,261]

In addition to the Bcl-family, reactive oxygen species also reportedly mediate the release of cytochrome c.[262,263] Reactive oxygen species generated from the mitochondrial electron transport chain induce cytochrome c dissociation from beef-heart submitochondrial particles via cardiolipin peroxidation.[264] In a model membrane, H_2O_2 induces release of cytochrome c from liposomes (a mimic of the mitochondrial membrane) by causing permeability transition.[30]

Reactive oxygen species are involved in apoptosis

The involvement of reactive oxygen species in the apoptotic signaling pathway is supported by the following observations: cells exposed to reactive oxygen species undergo apoptosis; apoptosis is often associated with generation of reactive oxygen species and/or depletion of antioxidants in the cell; and antioxidants protect the cell against apoptosis.

Cells exposed to reactive oxygen species undergo apoptosis

Reactive oxygen species are physiological metabolites, usually generated as intermediates in the reduction of oxygen. Mitochondria consume about 95% of the

inhaled oxygen and are the major source of reactive oxygen species in cells.[265,266] During transfer of electrons to molecular oxygen an estimated 1 to 5% of electrons in the respiratory chain in mitochondria lose their way, most participating in formation of superoxide. Anything that decreases the coupling efficiency of electron transport can increase production of superoxide anion.[225,267] The dismutation of superoxide anion by superoxide dismutase is the main source of H_2O_2 in mitochondria.[268,269] In the presence of transition metal ions, the hydroxyl radicals can be formed. Due to their higher reactivities relative to molecular O_2, these partially reduced metabolites of O_2 are often referred to as "reactive oxygen species".[270]

Although mitochondria are the main sources of reactive oxygen species, other sites also contribute to the oxidative stress. In the cytosol, for example, reactive oxygen species are generated through the arachidonic acid cascade when triggered by the Ca^{2+}-dependent enzyme, phospholipase A_2.[271,272] Some cytochrome P-450 isozymes can also produce superoxide anion.[273] The mitochondrial outer membrane enzyme monoamine oxidase catalyzes the oxidative deamination of biogenic amines and generates large amounts of H_2O_2, which contributes to an increase in the steady state concentration of reactive oxygen species within both the mitochondrial matrix and cytosol.[267] Reactive oxygen species are also generated during the respiratory burst in phagocytes such as neutrophils, macrophages, and monocytes.[274]

Cells exposed to reactive oxygen species undergo apoptosis, especially when their antioxidant capacities are surpassed.[28,29,275,276] An example of direct oxidant treatment causing apoptosis is that HL-60 cells exposed to low concentrations (15-50 μM) of H_2O_2

undergo apoptosis.[275] Under conditions that mimic the steady state of H_2O_2 *in vivo*, H_2O_2 can induce apoptosis at steady-state concentrations between 0.7 - 3 μM.[28] The steady-state concentration of H_2O_2 in cells is 10^{-9} to 10^{-7} under basal conditions (i.e. state III respiration).[277] This can increase about 4-fold in state IV respiration and 10-fold in the presence of a mitochondrial inhibitor (antimycin A) or redox agent (menadione) or in hyperoxia. Mitochondria generate 0.2-1.0 nmol H_2O_2/min/mg protein in state IV respiration, which represents 1-2% of oxygen consumption.[278,279,280,281,282,283] Under certain pathological circumstances, however, the concentration of H_2O_2 increase. For instance, plasma H_2O_2 levels in patients with diabetes can be higher than controls,[284] and in abscess fluid, the concentration of H_2O_2 reaches 100 μM.[285]

Different cell types have different tolerances for chronic H_20_2 exposure. Antunes and Cadenas[28] suggest that there might be one order of magnitude difference between the threshold value for apoptosis and the physiological concentrations of H_2O_2 in the cell. According to this suggestion, most cells should resist chronic H_2O_2 exposure if the concentrations are within ten times physiological concentrations.

H_2O_2 is an important reactive oxygen species in apoptosis models, due to its diffusibility and its proclivity for generating hydroxyl radicals via the metal catalyzed Fenton reaction.[268] Thus, metal chelators strongly protect against H_2O_2 induced apoptosis.[28] As mentioned before, hydroxyl radicals are arguably the most reactive chemical species produced in living organisms. They can attack a variety of cellular components, including nucleic acids, proteins, lipids, and other important biological molecules.[6,28,29,30,286,287,288]

Oxidative modifications of nuclei, proteins, and lipids have also been observed in cells undergoing apoptosis.[289,290,291]

Because of the importance of DNA for genetic inheritance, all organisms have evolved mechanisms to recognize and respond to DNA damage. If the damage is not severe, cells respond to the damage by undergoing cell cycle arrest to facilitate DNA repair.[292] If the damage is beyond safe repair, cells respond to the damage by undergoing apoptosis or necrosis.[292] Oxidation of intracellular proteins may modify either their function or structure to be recognized by other proteins. Proteinase inhibitors block thymocyte apoptosis, and probably they protect the same intracellular targets as those protected by antioxidants. Protein oxidation changes nuclear gene transcription such that the apoptotic pathway is activated.

Reactive oxygen species also play an important role in mediating apoptosis through affecting the reduction state of proteins. The oxidation state of cytochrome c is critical for the induction of nuclear changes associated with apoptosis. Specifically, only oxidized cytochrome c, not reduced cytochrome c, causes apoptosis. Such an apoptotic activity can be inhibited by cytochrome c reductase and selected antioxidants.[293]

H_2O_2 is a mediator in apoptosis,[294,295,296,297,298] and a signaling molecule involved in many cellular functions, such as oxidant-induced stress, signal transduction, development, and proliferation.[299,300,301,302,303] Therefore, the mechanism of apoptosis induced by H_2O_2 may be more complicated than we thought.

Other oxidants such as diamides,[304] semiquinones,[305] and superoxide anion[306] also mediate apoptosis. Most reactive oxygen species induce apoptosis, however, some reactive oxygen species might play special roles in apoptotic pathways. Superoxide anion, for example, plays an ambivalent role. On the one hand, superoxide anion acts directly as a pro-apoptotic reagent causing apoptosis. Thus some cells treated with external superoxide anion undergo apoptosis.[307,308,309] There is, moreover, a positive amplification loop between superoxide anion generation and mitochondrial damage during apoptosis.[310,311,312,313] In contrast, in various cells and tissues cytosolic Cu,Zn-SOD or mitochondrial Mn-SOD protect against apoptosis.[314,315,316,317] On the other hand, superoxide anion inhibits apoptosis.[318,319,320,321,322] For instance, when formed either via inhibition of Cu,Zn-SOD or treatment with xanthine/xanthine oxidase, superoxide anion attenuates Fas-mediated apoptosis by acting as reductant for cytochrome c.[318]

Apoptosis is often associated with generation of reactive oxygen species and depletion of antioxidants

The production of reactive oxygen species often increases before and during the apoptotic process.[314,323,324,325] For example, both ionizing and ultraviolet irradiation induce apoptosis and both generate reactive oxygen species such as H_2O_2 and hydroxyl radicals.[310] Also, increased production of reactive oxygen species results from the release of cytochrome c from mitochondria.[228,326] Release of cytochrome c from mitochondria during apoptosis interrupts the electron transport chain, resulting in the leaking of reactive oxygen species. In excitotoxic neuron death, for example, there is a secondary rise in superoxide production after the release of cytochrome c from mitochondria.[327]

Apoptosis is typically accompanied by a depletion of intracellular reduced glutathione (GSH),[289,328] and the depletion of glutathione pools is often concomitant with the increases in reactive oxygen species production.[329,330] GSH is the most rapid and abundant weapon against reactive oxygen species and regulates the oxidation state of many other cellular substances. Consequently, the glutathione reduction pair (GSH/GSSG) serves as an index of oxidative stress.[331] The release of cytochrome c from mitochondria is a cellular response to the depletion of glutathione, independent of the destiny of the cells, i.e., apoptosis or survival.[332]

Antioxidants protect cells against apoptosis

Antioxidants and thiol reductants, such as vitamin E,[333,334,335] vitamin C,[336] glutathione precursors S-adenosylmethionine and N-acetylcysteine,[337,338] overexpression of thioredoxin,[339] Mn-SOD,[340] and Bcl-2[341,342] can block or delay apoptosis. For example, vitamin E inhibits apoptosis of striatal neurons grown in culture and prevents neuronal degeneration induced by amyloid beta peptide in vitro. Vitamin C protects the vascular wall in patients with congestive heart failure via inhibiting endothelial cell apoptosis induced by tumor necrosis factor-alpha.[336]

Bcl-2 also can inhibit apoptosis via its antioxidant properties. For instance, Bcl-2-expressing cells do not undergo apoptosis or increase mitochondrial H_2O_2 production in response to apoptotic stimuli, while these cells have increased constitutive mitochondrial H_2O_2 generation and NAD(P)H availability.[341] Bcl-2 reverses viability defects of yeast

lacking superoxide dismutase and substantially improves survival of wild-type *S. cerevisiae* under death-inducing conditions.[342, 343] The Bcl-2 knockout mice demonstrate fulminant lymphoid apoptosis. These mice suffer from severe polycystic kidney disease and follicular hypopigmentation, both of which are the result of oxidative stress.[344,345] In addition, Bcl-2 allows cells to adapt to an increased state of oxidative stress, fortifying the cellular anti-oxidant defenses and counteracting the radical overproduction imposed by different cell death stimuli. In addition, Bcl-2 can block apoptosis through the modulation of antioxidant GSH.[346,347] This concept is supported by the observation that Bcl-2 rescues the GT1-7 neural cell line via blocking glutathione depletion.[348] How the expression of Bcl-2 increases the levels of intracellular GSH, however, is not clear.

It is particularly difficult to untangle cause from effect in the arena of apoptosis. Oxidant stress can clearly contribute to the release of cytochrome c from the mitochondria during apoptosis. Positive feedback mechanisms might be involved in the release of cytochrome c. H_2O_2 (and thus presumably peroxidation) is commonly used to induce apoptosis. Concomitantly the release of cytochrome c clearly promotes peroxidations, particularly if the cytochrome c undergoes scission. The calcium-activated proteases are the most plausible candidates as agents for the conversion of cytochrome c to more active peroxidatic agents. However, oxidant stress is not the only factor that contributes to release of cytochrome c. Other apoptotic stresses such as DNA damage also induce the release of cytochrome c.[349] Instead of functioning separately, oxidant stress and other factors might act together during apoptosis.

Purpose

The dual discoveries of the roles of Cu,Zn-SOD in familial amyotrophic lateral sclerosis and of cytochrome c in apoptosis have dramatically reframed our understanding of these enzymes. Let us highlight the main points made in this introduction as they relate to the need for further research. Amyotrophic lateral sclerosis is a disease in which a some patients have mutations of Cu,Zn-SOD. Many mutations of Cu,Zn-SOD alter the Zn-binding site. In addition, H_2O_2 can further damage the Zn-binding site. Current views favor the hypothesis that the mutant superoxide dismutase experiences a 'gain-of-function' that is associated with the etiology of familial amyotrophic lateral sclerosis. Thus, exploring the impact of removing zinc from the protein on potential toxic pro-oxidant activity of the enzyme in the presence of H_2O_2 may provide a clue regarding the 'gain-of-function' of superoxide dismutase in familial amyotrophic lateral sclerosis.

Cytochrome c plays an important role in assisting the production of ATP in the mitochondrial electron transport chain. Its release from mitochondria, however, triggers apoptosis. Although the mechanism of release of cytochrome c from mitochondria is not clear, reactive oxygen species such as H_2O_2 can play a role. H_2O_2 and its interaction with cytochrome c may transform cytochrome c from a life supporter to a cell killer. Studies of the peroxidatic activity of cytochrome c and the factors affecting this activity may provide important information for answering this question.

Another avenue for investigation is to examine the interaction of the peroxidatic activities of superoxide dismutase and cytochrome c in combination. Amyotrophic lateral sclerosis

seems to be a multifactor disease involving the mutation of Cu,Zn-SOD, malfunction of mitochondria, generation of reactive oxygen species, and the release of cytochrome c from mitochondria. The interaction of these factors might cause the disease by permitting and enhancing each other, or even forming a positive feedback loop in their interactions. Apoptosis is involved in the neuron death of amyotrophic lateral sclerosis and release of cytochrome c triggers apoptosis. Therefore, instead of examining separately, exploring the interaction between mutant Cu,Zn-SOD and cytochrome c might yield some interesting results.

MATERIALS AND METHODS

Reagents

Copper, zinc superoxide dismutase (from bovine erythrocytes), manganese superoxide dismutase (from *Escherichia Coli*), cytochrome c (from horse heart, formerly listed as Sigma type VI), benzoic acid (sodium salt), sodium formate, potassium phosphate (monobasic: anhydrous), sodium fluoride, ferric chloride (anhydrous), L-ascorbic acid (sodium salt), diethylenetriaminepentaacetic acid, and Coomassie brilliant blue R staining solution were purchased from Sigma Chemicals. Ethylenediaminetetraacetate (disodium salt), sodium bicarbonate, sodium acetate, cupric sulphate, and D-mannitol were purchased from Fisher Chemicals Inc. Hydrogen peroxide, sodium chloride, zinc sulphate, and ferrous sulphate were obtained from BDH Chemicals. N,N,N',N'-Tetramethyl-1,4-phenylenediamine (dihydrochloride salt), calcium chloride, and potassium phosphate (dibasic: crystals) were obtained from Aldrich, Mallinkrodt, and Allied Chemicals, respectively. Desferrioxamine (desferal mesylate) was a gift from CIBA Pharmaceuticals (now Novartis Pharmaceuticals Canada Inc.). An anthocyanin mixture, and purified cyanidin-3-O-beta-glucopyranoside were purchased from Polyphenols Laboratories (Norway). Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carbonic acid) was from OxisResearch.

All solutions were prepared using distilled and deionized water, except in the preparation of TMPD, where potassium phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA was used. All reactions were carried out in potassium phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA, except for the reactions whose objective was to study the

effect of different buffers, buffer concentration, pH, and EDTA. TMPD stock solution (30 mM) was freshly prepared daily with potassium phosphate buffer (both the buffer and TMPD were saturated with argon gas and continuously bubbled with argon to prevent its auto-oxidation during the experiments).

Preparation of Zn-depleted superoxide dismutase*

Cu,Zn-SOD apoprotein was made through extensive dialysis against 10 mM EDTA, in 50 mM acetate at pH 3.8 by following the method previously described, without trying to improve upon or calibrate it.[3] The chelating agent was removed through dialysis against 100 mM NaCl in the same buffer[350] and then against acetate buffer alone. The resulting apoprotein was incubated with a 10% excess of cupric sulphate per mol subunit of enzyme overnight at 4 °C, and 1 to 2h dialysis against 5 mM sodium acetate (pH 3.5-4.0) to restore the copper moiety.[123] Depletion of zinc from the dialyzed Cu,Zn-SOD was assumed to be similar to that reported, but in future studies Zn-depletion should be confirmed by trace element analysis using inductively coupled plasma emission spectrophotometry or atomic absorption spectroscopy.

* The molecular weight of modified Cu,Zn-SOD could be verified by SDS-PAGE or protein mass spectroscopy, and the metal content of chemically modified Cu,Zn-SOD can be checked by inductively coupled plasma emission spectrophotometry (ICP), or atomic absorption spectroscopy (AA). The ICP spectrophotometer utilizes a plasma beam to excite elemental electrons, which produce photons unique to each element. Using stoichiometric techniques, elemental concentrations can be converted into molecular weight percentages. The AA technique utilizes the observation that certain wavelengths of radiation emitted by excited atoms are strongly absorbed by unexcited atoms of the same element (emission spectrum). The radiation will be reduced if it passes through an area containing such unexcited atoms. This reduction can be measured by a detector, and the concentration of the element of interest can be calculated. Since samples are usually liquids or solids, the atoms or ions to be analyzed must be vaporized in a flame or graphite furnace. The atoms absorb ultraviolet or visible light and make transitions to higher electronic energy levels. The metal concentration is determined from the amount of absorption.

SDS-PAGE

Sample preparation: 25 µl of cytochrome c (1.2 mM) and 20 µl of H_2O_2 (750 mM) were pre-incubated for 10 min at 20°C before the reagents of interest were added. This time was chosen as the moment where peroxidatic activity stabilized somewhat. The total volume of the mixture was then brought up to 3 ml with potassium phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA. The concentrations shown on the legend were the final concentrations in the tube. After the solutions were thoroughly mixed, 15 µl of sample was taken from the tube, mixed with 15 µl of 2x loading buffer, heated for 5 min at 95°C, and then cooled on ice for 5 min prior to loading on the gel.

SDS-PAGE electrophoresis apparatus was a Bio-Rad Mini-Gel System using 12% acrylamide gel. Electrophoresis was carried out in SDS-PAGE running buffer (1 L solution contained 3.028g Tris, 14.413g glycine and 10 ml 10% SDS, pH 8.3) under 80 V at 20°C until the front dye ran off the gel (about 2.5 hours). The gel was stained with Coomassie Brilliant Blue R Staining Solution for 1 hour on a horizontal shaker, and then de-stained with de-staining buffer (which contained 45% methanol, 10% acetic acid, and 40% double distilled H_2O) for 4 hours on a horizontal shaker before the picture was taken.

Assay procedure

For TMPD peroxidation activity, all of the reagents (except TMPD) were added to a cuvet, which was then filled with buffer to 2.96 ml. 40 µl of TMPD stock solution was added to initiate the reaction in a final volume of 3 ml. If the reactions needed incubation, the stock reagents were incubated in the cuvet for 10 min and then the buffer and TMPD were added to initiate the reaction. If not otherwise stated, cytochrome c was preincubated with 5 mM H_2O_2 (final concentration after dilution with cytochrome c and buffer) in the cuvet for 10 min before initiating the reaction. The concentrations shown

on the legend were the final concentrations in the cuvet. The oxidation of TMPD was followed at 610 nm, with a Beckman DB-GT spectrophotometer. The spectrophotometer was equipped with a Beckman 10 inch strip chart recorder and facilities for online data acquisition by a PC 486 computer. Initial rates were obtained by calculating the slopes of the initial portions of each reaction. The pH was checked at the start and upon the completion of the reaction for the representative conditions to ensure that there is no change of pH during the reaction.

Autoxidation of TMPD under the conditions studied was slow enough that generation of the chromophore even under aerobic conditions could be attributed purely to the peroxidatic reaction. In the reactions with test substances, I always measured autoxidation to confirm that this was not interfering with our assessment of peroxidation. Except for one circumstance (the presence of benzoate), autoxidation did not interfere. Background TMPD peroxidation was corrected for: peroxidatic activity of superoxide dismutase represents the net peroxidatic activity after subtracting the generation of chromophore in the presence of all reactants except superoxide dismutase. I also confirmed that metal chelator DTPA had no effect on baseline TMPD peroxidation.

TMPD as an indicator of peroxidatic activity

The index for peroxidatic activity used in this study was TMPD oxidation. TMPD is a single-electron reducing agent. Its reduced form is colorless in aqueous solution, and its oxidized form, the "Wurster's blue" radical cation, has a stable blue color with a peak absorbance at 610 nm. The chromogen generated in the oxidation of TMPD can be used

as an indicator of a wide range of either autoxidations or peroxidatic oxidations. TMPD has significant advantages that have led to its widespread use in both biochemical and histochemical procedures. These include: (1) TMPD does not interfere with most biological chromophores. (2) The peak absorbance of the oxidized form of TMPD is at relatively long wavelengths, making it relatively less sensitive to turbidity changes. (3) The products of TMPD oxidation do not undergo further reactions of quinonoid products, e.g., polymerizations or Diels-Alder condensations.

Statistical approaches

At the start of experimentation, two-way analysis of variance initially seemed the method of choice. ANOVA seemed appropriate for assessing the peroxidatic activity of Cu,Zn-SOD as modified by cytochrome c, because both native and Zn-depleted superoxide dismutase were used. The differences in TMPD peroxidation within, as well as among, the following groups needed to be tested: *with* and *without* native or Zn-depleted superoxide dismutase in the *absence* and in the *presence* of cytochrome c.

When the studies showed that Cu,Zn-SOD had no obvious peroxidatic activity and no synergism with cytochrome c, it became clear that for simply testing the differences between native and Zn-depleted superoxide dismutase, two-way analysis did not provide any advantages over simpler statistical methods. For studies of the peroxidatic activity of cytochrome c and factors affecting it, two-way analysis of variance was similarly unnecessary. The main task of this study related in the end, to problems associated with

inference based on "small" samples, Student's t-Test was therefore adopted as the statistical method to reject or accept the null hypothesis.

Each experiment was repeated on at least two different days. Moreover, the measurements on a given day were made in duplicate or triplicate. For every experiment, all appropriate controls were run with every treatment group for internal comparisons. As a result, some controls were repeated daily through the experimental period to provide an accurate estimate of variability and a consistent basis for making comparisons across experiments. The mean for each day was calculated by averaging replicates in a given experiment with number of repetitions on that day. The standard errors of the means were calculated using standard statistical methods with number of repeated days as n. The level of confidence required for significance was selected in advance to be $p < 0.05$.

RESULTS

Section 1 : Peroxidatic activity of superoxide dismutase†

TMPD oxidation is accelerated by H_2O_2 but not Cu,Zn-SOD

TMPD autoxidation was insignificant (Figure 1) in comparison with its peroxidation (or peroxidatic oxidation --- oxidation in the presence of H_2O_2). After a brief induction period, autoxidation was linear until one or more reactants began to be exhausted. Adding Cu,Zn-SOD did not accelerate TMPD autoxidation (data not shown).

Figure 2 shows a progress curve for TMPD peroxidation. Addition of Cu,Zn-SOD had no effect on either TMPD oxidation or TMPD peroxidation, regardless of whether Cu,Zn-SOD was added before or after H_2O_2 (Figure 3 and 4).

Peroxidatic activity of native Cu,Zn-SOD is detectible only at high concentrations

Peroxidation was detectable only at extremely high concentrations of Cu,Zn-SOD: 500 U/ml and 1000 U/ml (Fig. 5). This response was dose-dependent, but concentrations were much higher than needed to scavenge any conceivable concentration of superoxide.

Bicarbonate confers peroxidatic activity on Cu,Zn-SOD

The addition of 10 mM bicarbonate increased the peroxidatic activity of Cu,Zn-SOD toward TMPD by 60% (Fig. 6), confirming earlier results by other researchers.[21] The

† Unless specified, superoxide dismutase always refers to Cu,Zn-SOD.

bicarbonate is believed to relay the free radical generated in the active site of the enzyme to the outside.

Removing zinc from Cu,Zn-SOD increases its peroxidatic activity toward TMPD

To study the postulated role of a weakened zinc crevice in the peroxidatic activity of Cu,Zn-SOD, I used Zn-depleted Cu,Zn-SOD[‡], which gave significant peroxidatic activity. Removing zinc from Cu,Zn-SOD increased the peroxidatic activity of superoxide dismutase by 8-fold toward a level approaching that achieved by the addition of bicarbonate (Fig. 6). While adding 10 mM bicarbonate increased the peroxidatic activity of Zn-depleted superoxide dismutase more than two fold, it is noteworthy that the peroxidatic activity in this situation was not significantly different from that obtained with native superoxide dismutase.

[‡]Please note that the zinc content of dialyzed product was not determined. If this work were to be extended, the Zn-depleted enzyme would be sent for analysis of zinc content by AA.

Figure 1. TMPD autoxidation.

TMPD autoxidation (TMPD with ambient oxygen) was initiated by adding TMPD. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentration of TMPD was 400 μM. The data plotted were from three different days. Error bars represent standard errors of respective means.

Figure 1
TMPD Autoxidation

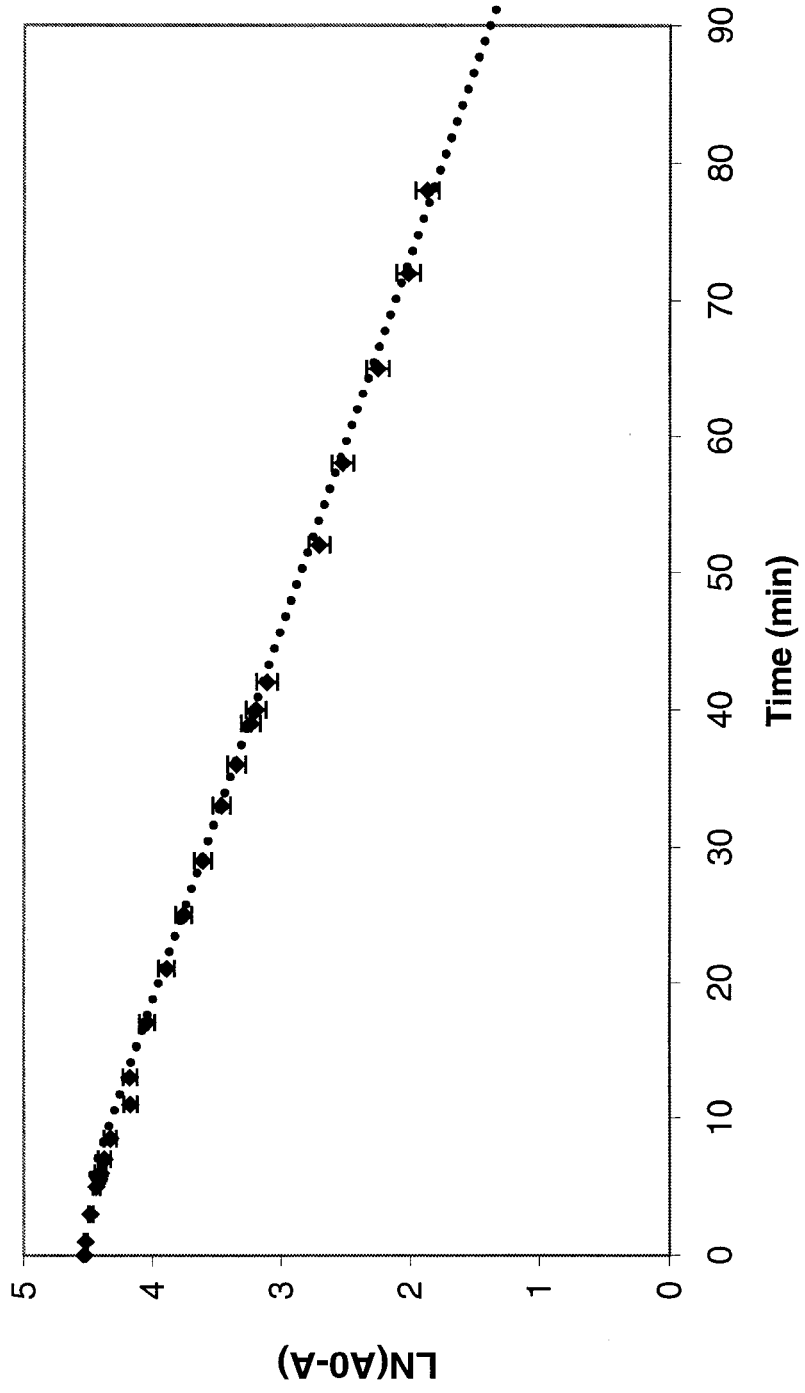


Figure 2. TMPD oxidation with addition of H_2O_2.

TMPD peroxidation was initiated by adding TMPD and then H_2O_2. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentration of TMPD was 400 μM and final concentration of H_2O_2 was 15 mM. The data plotted were from three different days. Error bars represent standard errors of respective means.

Figure 2
TMPD oxidation with addition of H_2O_2

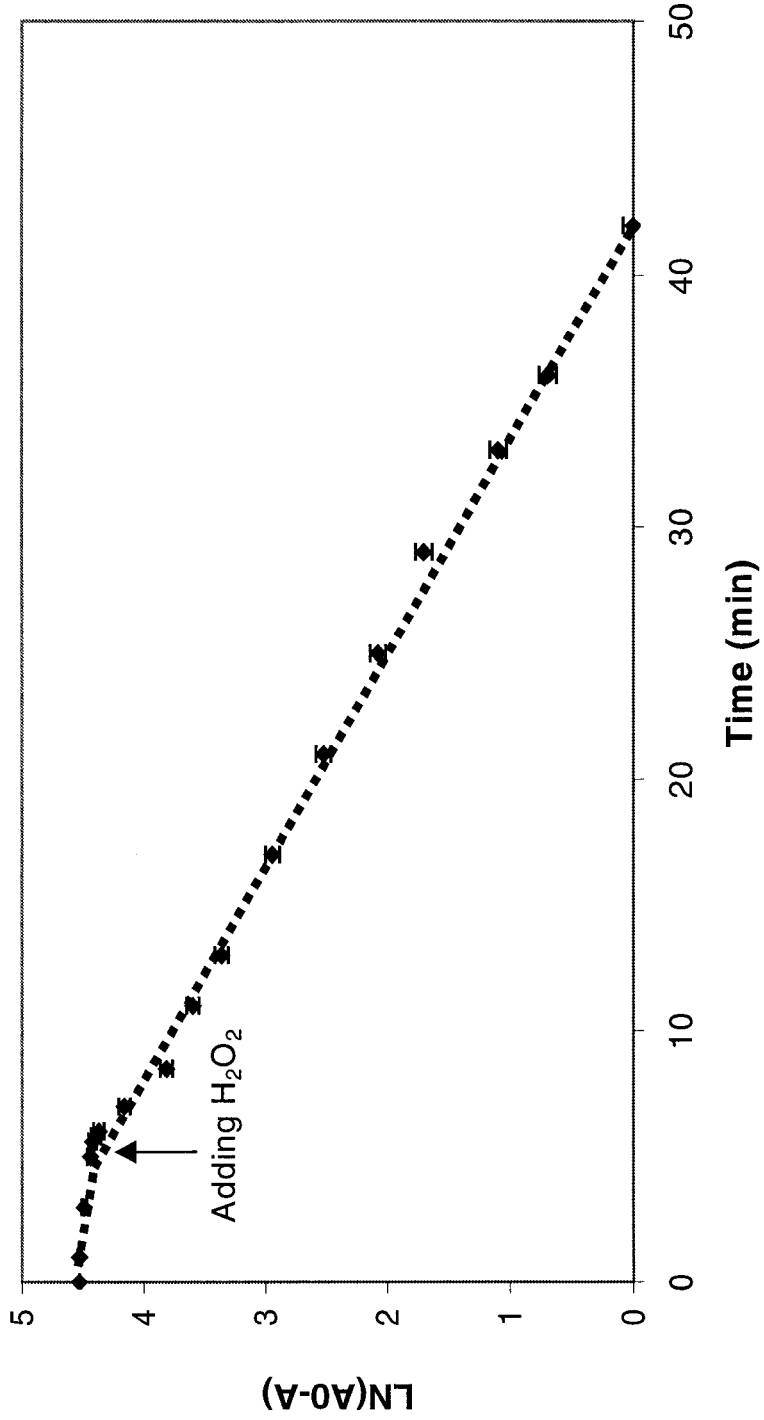


Figure 3. TMPD oxidation with addition of Cu,Zn-SOD and H_2O_2.

TMPD oxidation was initiated by adding TMPD. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentration of TMPD was 400 μM and final concentrations of Cu,Zn-SOD and H_2O_2 were 3 U/ml and 15 mM, respectively. The data plotted were from three different days. Error bars represent standard errors of respective means.

Figure 3
TMPD oxidation with addition of Cu,Zn-SOD and H_2O_2

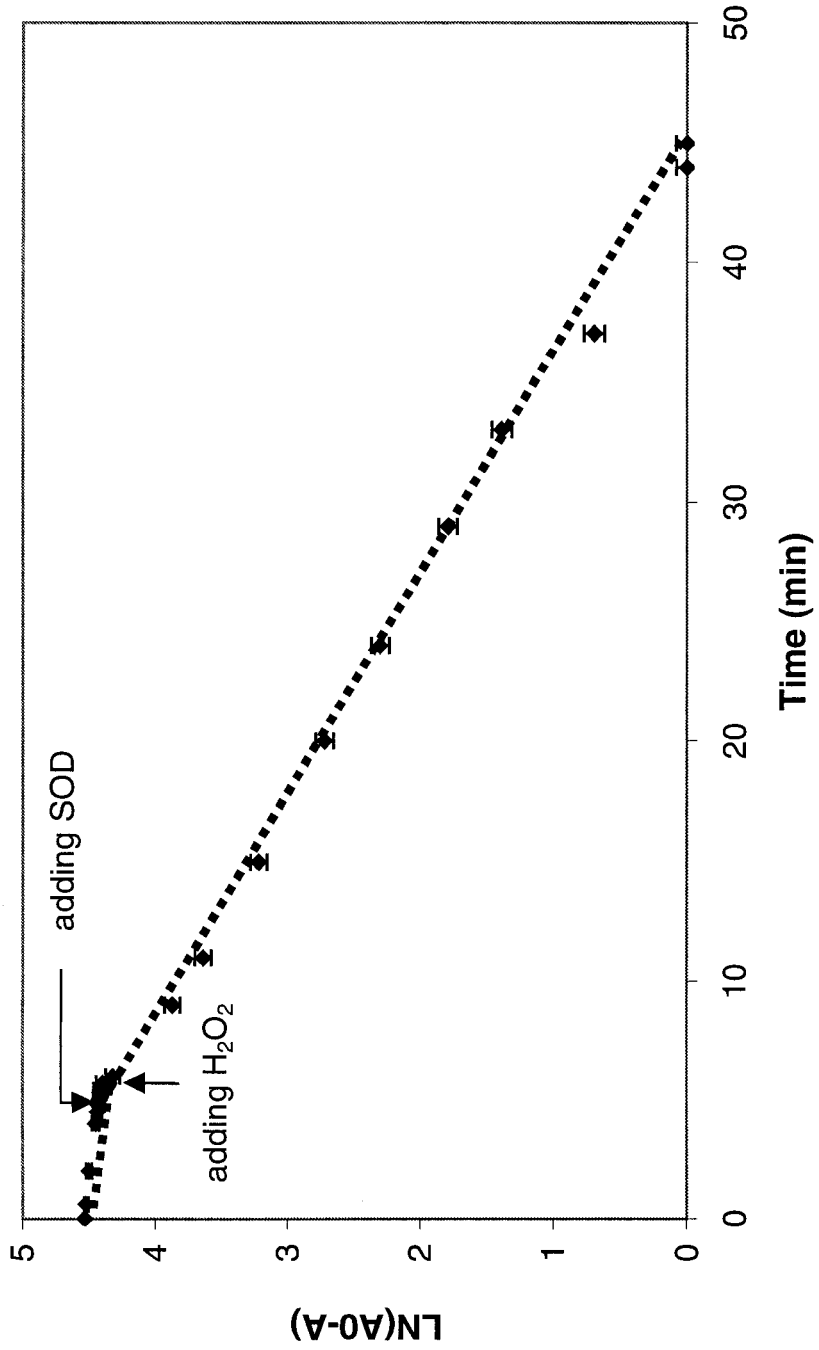


Figure 4. TMPD oxidation with addition of H_2O_2 and Cu,Zn-SOD.

TMPD oxidation was initiated by adding TMPD. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentration of TMPD was 400 μM and final concentrations of H_2O_2 and Cu,Zn-SOD were 15 mM and 3 U/ml, respectively. The data plotted were from three different days. Error bars represent standard errors of respective means.

Figure 4
TMPD oxidation with addition of H_2O_2 and Cu,Zn-SOD

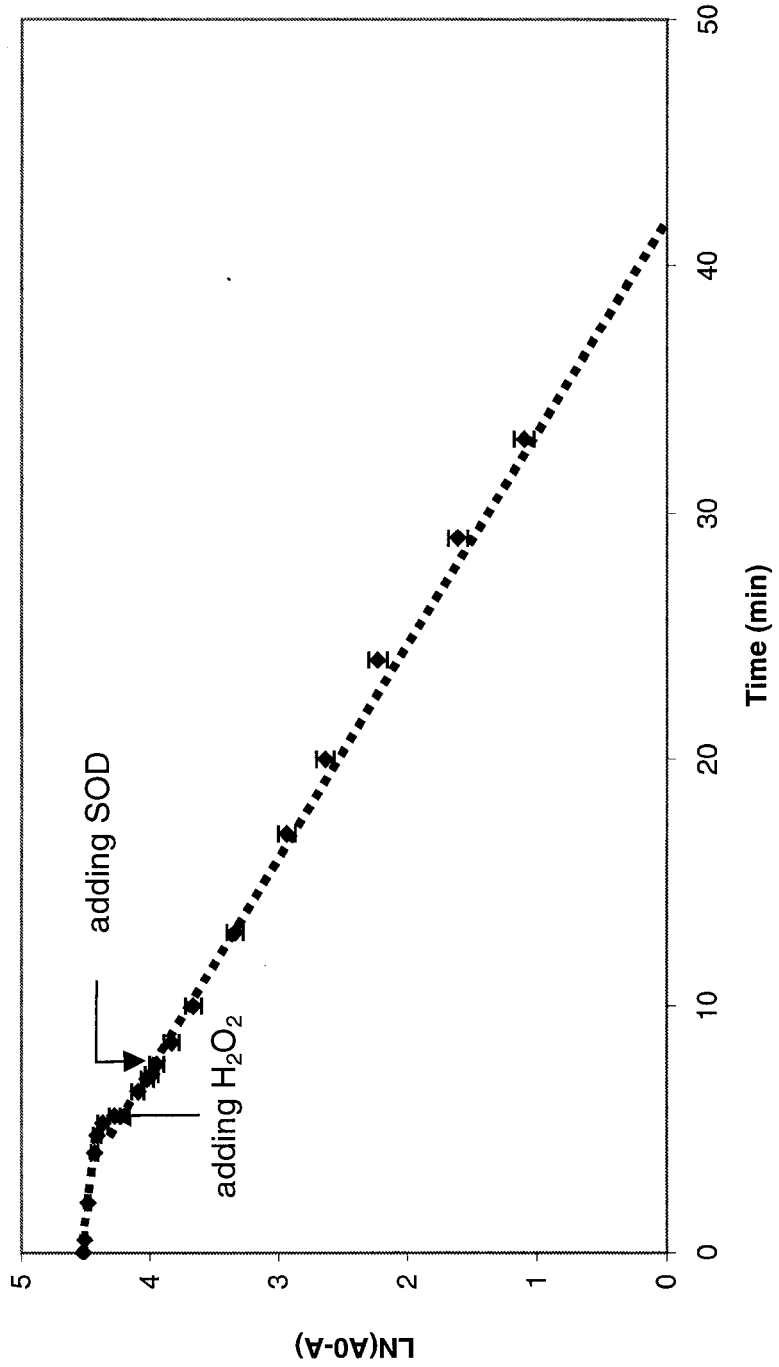


Figure 5. Dose response curve for peroxidatic activity of native Cu,Zn-SOD.

The stock reagents (H_2O_2, 750 mM; and Cu,Zn-SOD, 10000 U/ml) were incubated for 10 min in the cuvet, and then buffer and TMPD were added to initiate the reaction. The buffer was 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4). The reactions were conducted at 20°C in a total volume of 3 ml. The final concentrations of TMPD and H_2O_2 were 400 μM and 5 mM, respectively. The final concentrations of Cu,Zn-SOD were as indicated in the figure. The data plotted were from two different days. Error bars represent standard errors of respective means.

Figure 5
Dose response curve for peroxidatic activity of native Cu,Zn-SOD

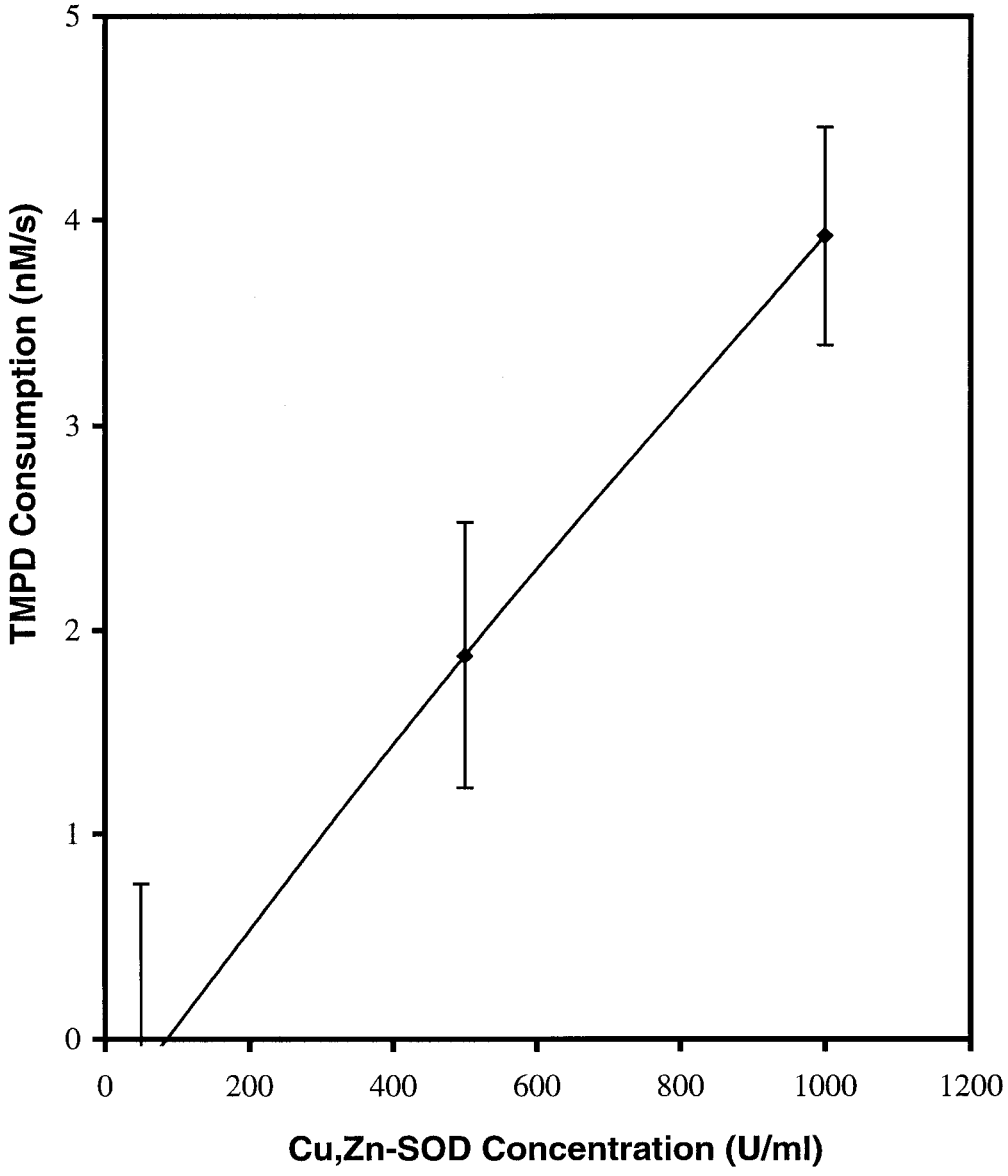
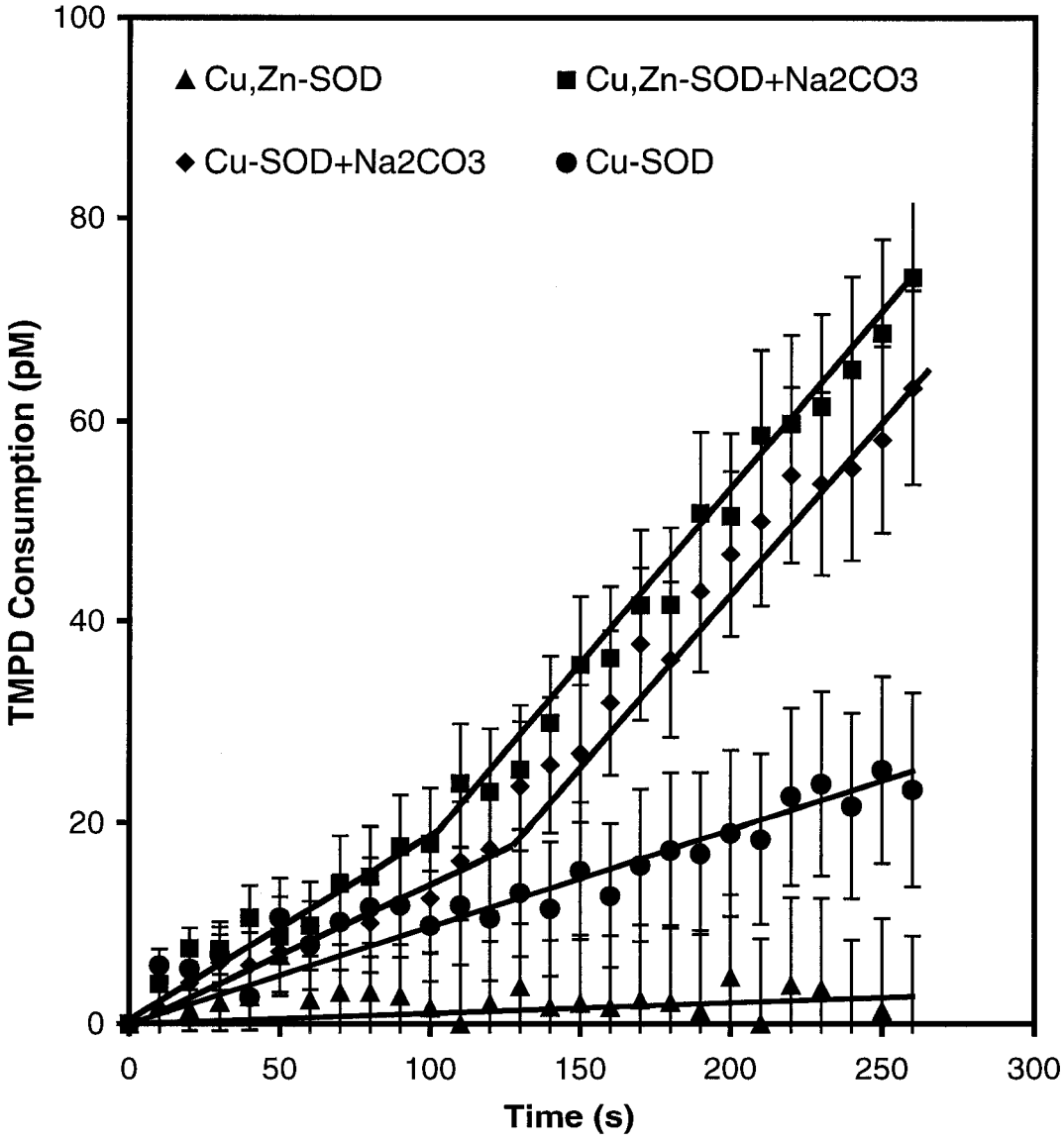


Figure 6. Effect of bicarbonate on peroxidatic activity of superoxide dismutase.

The stock reagents (H_2O_2, 750 mM; Cu,Zn-SOD, 10000 U/ml; or Cu-SOD, 10000 U/ml) were incubated for 10 min in the cuvet, then buffer and TMPD were added to initiate the reaction. The buffer was 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4). The reactions were conducted at 20°C in the absence and presence of bicarbonate and both native and Zn-depleted Cu,Zn-SOD were tested. Total volume of each reaction mixture was 3 ml. The final concentrations of TMPD, H_2O_2, Na_2CO_3, Cu,Zn-SOD, and Cu-SOD were 400 μM, 5 mM, 10 mM, 50 U/ml, and 50 U/ml, respectively. The data plotted were from two different days. Error bars represent standard errors of respective means.

Figure 6
Effect of bicarbonate on peroxidatic activity of SOD



There is no synergism between the peroxidatic activities of Cu,Zn-SOD and cytochrome c

To explore the potential synergism between the peroxidatic activities of Cu,Zn-SOD and cytochrome c, I added these to the reaction mixture separately, and together. The data, however, did not show any synergism. Adding 10 mM bicarbonate, and/or removing zinc from superoxide dismutase, did not generate the synergism either (Figures 7, and 8).

In the discussion section, I will return to the following points: the release of peroxidatic activity of Cu,Zn-SOD by small molecules, and potential consequences of removing zinc from the protein.

Section 2: Peroxidatic activity of cytochrome c

Characterization of the reaction

TMPD oxidation is a slow reation in the absence of H_2O_2 or/and cytochrome c

Figure 9 shows a typical reaction profile for the progress of TMPD oxidation, TMPD peroxidation, and TMPD peroxidation induced by H_2O_2 pre-incubated cytochrome c. Unless otherwise specified, TMPD peroxidation induced by H_2O_2 pre-incubated cytochrome c refers to cytochrome c-catalyzed TMPD peroxidation. TMPD peroxidation in absence of cytochrome c refers to uncatalyzed TMPD peroxidation; and cytochrome c refers to H_2O_2 pre-incubated cytochrome c. According to the slope of the reaction, oxidation of TMPD was very slow ($k1 = 5.2 \times 10^{-5}\ s^{-1}$), and the rate of uncatalyzed TMPD peroxidation was 3.4 times greater than the rate of TMPD oxidation. Compared to

uncatalyzed TMPD peroxidation, the reaction rate of cytochrome c-catalyzed TMPD peroxidation increased by a further 9.6 fold.

Cytochrome c-catalyzed TMPD peroxidation is the first order with respect to time and second order with respect to TMPD

The cytochrome c-catalyzed TMPD peroxidation was first order with respect to time (Figure 10). The apparent first order rate constant was therefore used to quantify velocities and compare reactions.

In contrast, the cytochrome c-catalyzed TMPD peroxidation was second order with respect to TMPD (Figure 11) and catalysis by cytochrome c displayed classic Michaelis-Menten saturation kinetics (Figures 12 and 13), with the K_m for H_2O_2 being 8.5 mM.

Cytochrome c changes the temperature profile for the TMPD peroxidation

The Arrhenius plot for uncatalyzed TMPD peroxidation yielded a straight line (Figure 14). The activation energy and entropy of activation of the reaction were calculated to be 10.6 kcal.mole^{-1} and ΔS^*_{298} = -11.4 cal K^{-1} mol^{-1}. These come from the logarithmic form of the Arrhenius Equation [$\log k = \frac{-Ea}{2.303RT} + \log A$], where k is the rate constant, **A** is frequency factor, $\mathbf{E_a}$ is activation energy, **R** is universal gas constant, T is absolute temperature. Graphically, log k is the Y axis, -Ea/2.303R is the slope, 1/T is X axis, and log A is the intercept.

The Arrhenius plot for cytochrome c-catalyzed TMPD peroxidation, however, was biphasic (Figure 14), yielding a different activation energy at different temperatures. On

this basis, between 22.5°C and 25°C the activation energy was 4.0 kcal.mole^{-1}, whereas between 50°C and 52.5°C it was 17.1 kcal.mole^{-1}.

Effects of buffer on cytochrome c-catalyzed TMPD peroxidation

Peroxidatic activity of cytochrome c is higher in EDTA-phosphate buffer

Effect of buffer on cytochrome c-catalyzed TMPD peroxidation not only depends on the type of buffer, but also depends on whether the buffer contains EDTA. When the reaction was conducted in HEPES buffer containing 0.1 mM EDTA, the reaction rate decreased almost 30% when compared with the reaction conducted in potassium phosphate buffer that also contained 0.1 mM EDTA. The reaction rate, however, decreased by 76% if conducted in potassium phosphate buffer containing no EDTA when compared with the reaction conducted in the same buffer containing 0.1 mM EDTA (Figure 15).

Optimum pH for cytochrome c-catalyzed TMPD peroxidation is around 8

The effect of pH on cytochrome c-catalyzed TMPD peroxidation revealed a slight increase in reaction rate up to pH 8, followed by a precipitous fall as the pH increased further (Figure 16). The decrease in reaction rate above pH 8 is consistent with a previous report that the rate of the reaction between ferrocytochrome c and H_2O_2 decreases as the pH increases above pH 7.9.[209]

Figure 7. Combined peroxidatic activities of Cu,Zn-SOD and cytochrome c.

The stock reagents (H_2O_2, 750 mM; Cu,Zn-SOD, 10000 U/ml; and cytochrome c, 1.2 mM) were incubated for 10 min in the cuvet, and then buffer and TMPD were added to initiate the reaction. The buffer was 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4). The reactions were conducted at 20°C in the absence and presence of bicarbonate in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, Na_2CO_3, Cu,Zn-SOD, and cytochrome c were 400 μM, 5 mM, 10 mM, 50 U/ml, and 10 μM, respectively. The data plotted were from two different days. Error bars represent standard errors of respective means.

Figure 7
Combined peroxidatic activities of Cu,Zn-SOD and cytochrome c

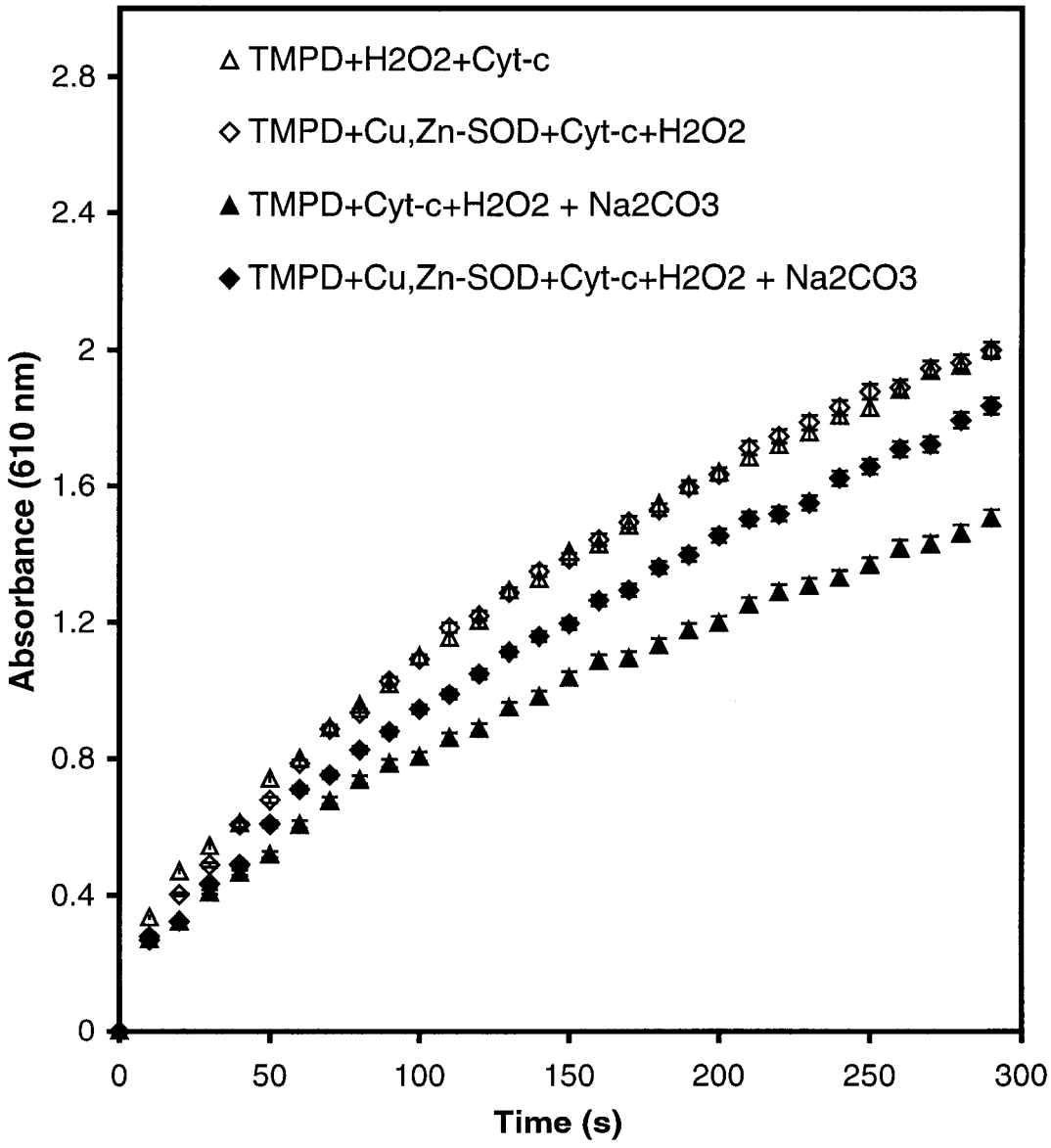


Figure 8. Combined peroxidatic activities of Cu-SOD and cytochrome c.

The stock reagents (H_2O_2, 750 mM; Cu-SOD, 10000 U/ml; and cytochrome c, 1.2 mM) were incubated for 10 min in the cuvet, and then buffer and TMPD were added to initiate the reaction. The buffer was 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4). The reactions were conducted at 20°C in the absence and presence of bicarbonate in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, Na_2CO_3, Cu-SOD, and cytochrome c were 400 μM, 5 mM, 10 mM, 50 U/ml, and 10 μM, respectively. The data plotted were from three different days. Error bars represent standard errors of respective means.

Figure 8
Combined peroxidatic activities of Cu-SOD and cytochrome c

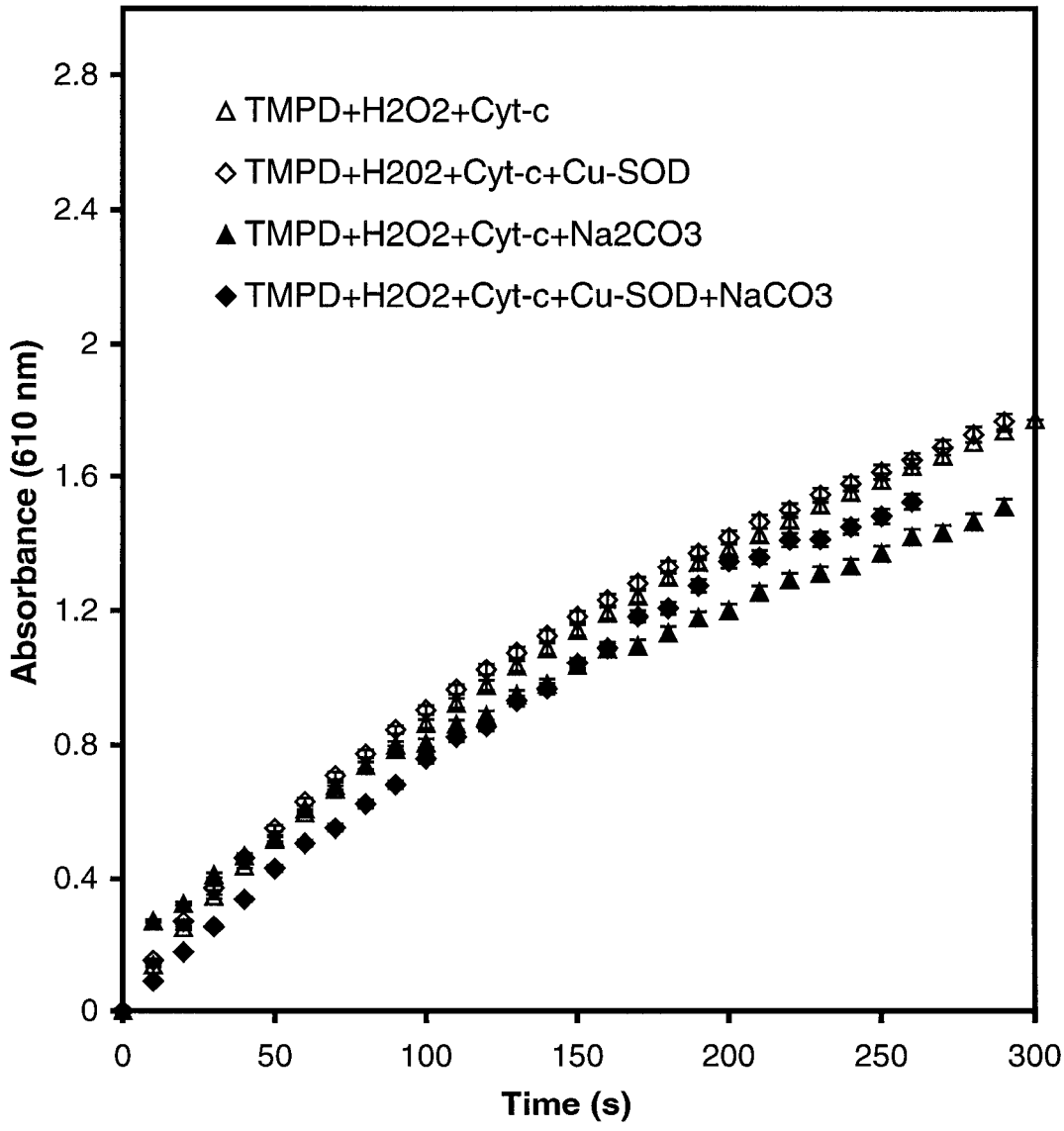


Figure 9. TMPD peroxidation: Effect of cytochrome c.

The reagents of stock solution (H_2O_2, 750 mM; and cytochrome c, 1.2 mM) were added to the cuvet, and then buffer and TMPD were added to initiate the reaction. The buffer was 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4). In cytochrome c-catalyzed TMPD peroxidation, 25 µl of cytochrome c stock solution was incubated with 20 µl of H_2O_2 stock solution for 10 min in the cuvet, and then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The plots for TMPD alone, TMPD plus cytochrome c, and TMPD plus H_2O_2 represent control reactions. The data plotted were from three different days. Error bars represent standard errors of respective means.

Figure 9
Peroxidatic oxidation of TMPD:
Effect of cytochrome c

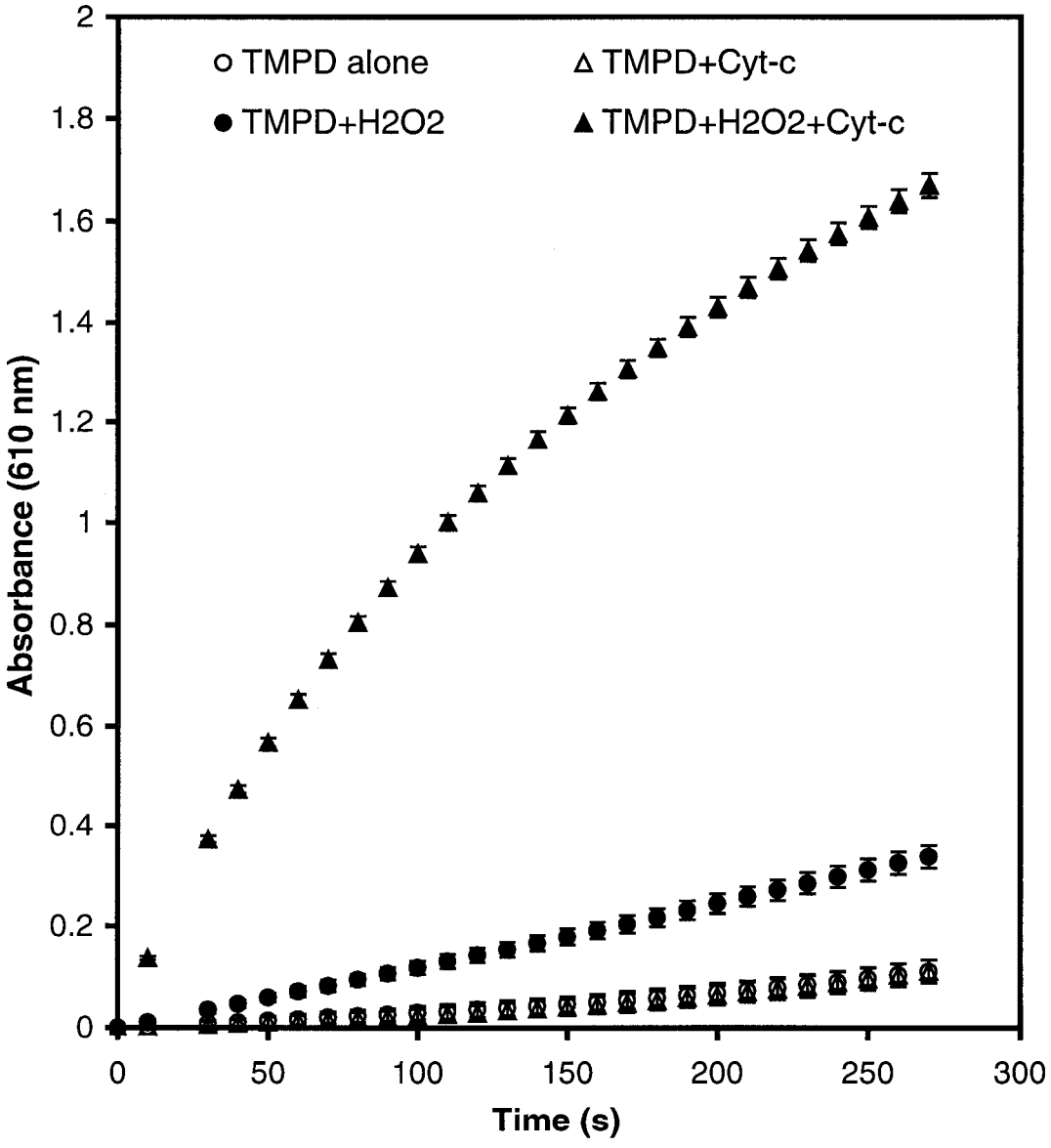


Figure 10. Cytochrome c-catalyzed TMPD peroxidation

25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and then buffer and TMPD were added to initiate the reaction. The buffer was 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4). The reactions were conducted at 20°C in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The data plotted were from three different days. Error bars represent standard errors of respective means.

Figure 10
Cytochrome c-catalyzed TMPD peroxidation

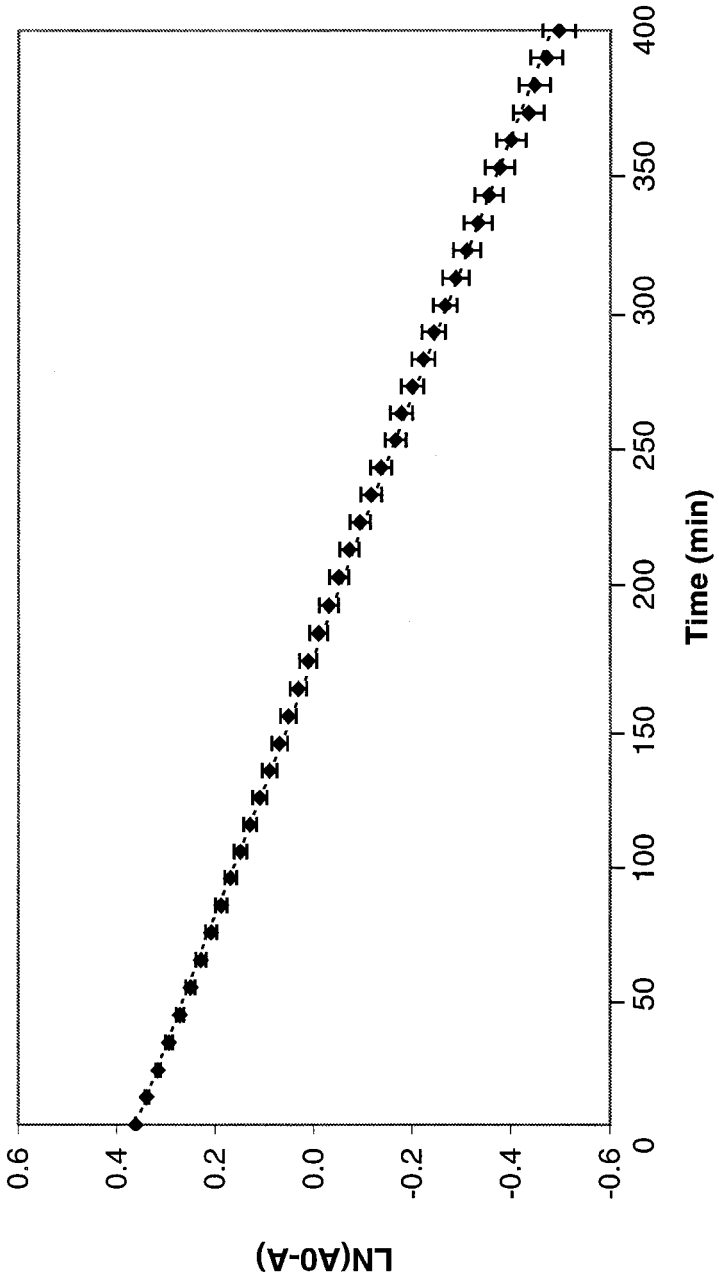


Figure 11. Cytochrome c-catalyzed TMPD peroxidation: Effect of TMPD concentration.

Double-logarithm plot of reaction velocity as a function of TMPD concentration. 25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and then buffer and stated amount of TMPD were added to initiate the reaction. The buffer was 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4). The reactions were conducted at 20°C in a total volume of 3 ml. The final concentrations of H_2O_2 and cytochrome c were 5 mM and 10 µM, respectively. The data plotted were from two different days.

Figure 11
Cytochrome c-catalyzed TMPD Peroxidation:
Effect of TMPD concentration

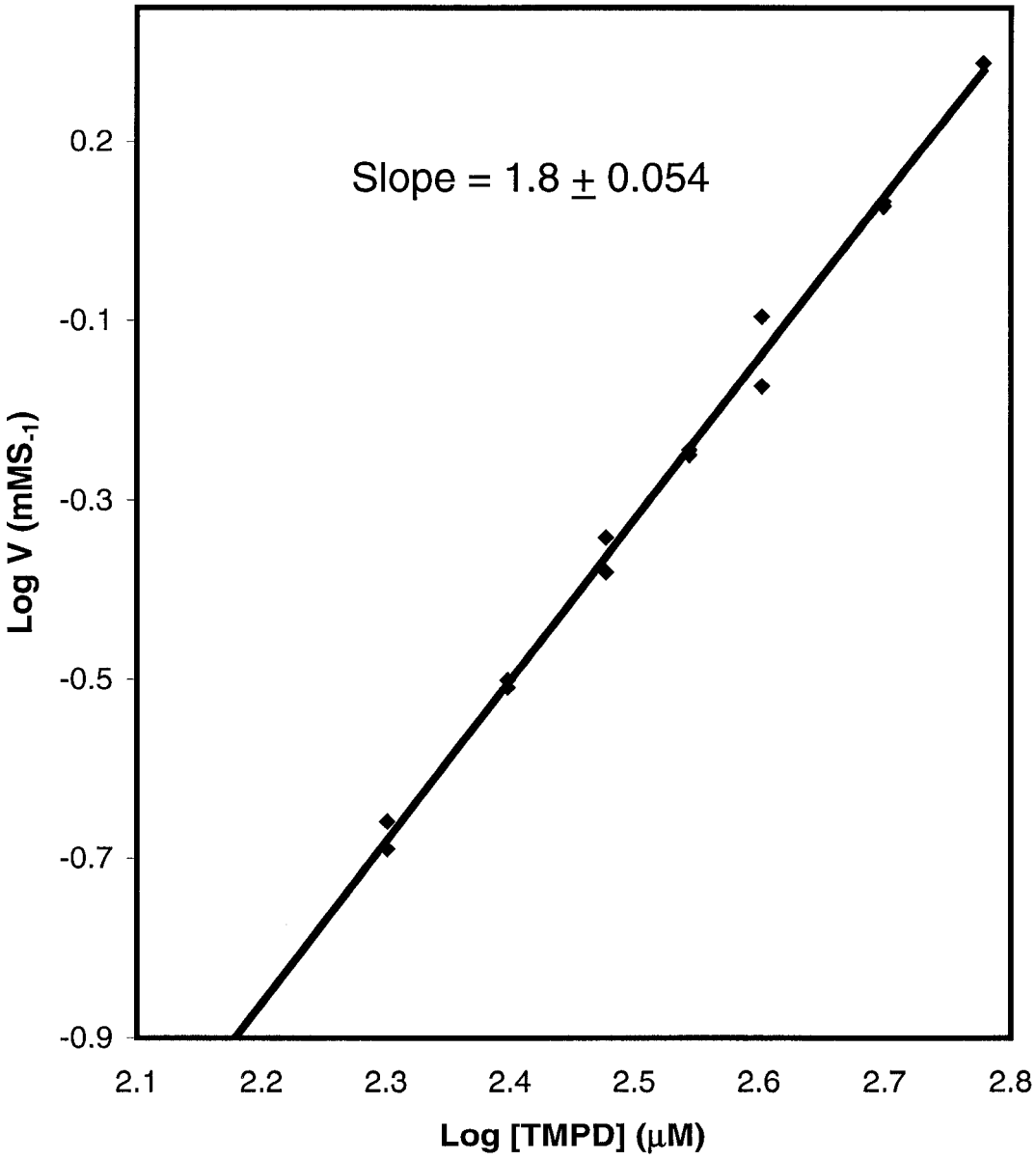


Figure 12. Cytochrome c-catalyzed TMPD peroxidation: Effect of cytochrome c concentration.

Double-reciprocal plot of reaction velocity as a function of cytochrome c concentration. Cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and then buffer and TMPD were added to initiate the reaction. The buffer was 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4). The reactions were conducted at 20°C in a total volume of 3 ml. The final concentrations of TMPD and H_2O_2 were 400 µM and 5 mM, respectively. The final concentrations of cytochrome c were as indicated in the figure. The data plotted were from two different days.

Figure 12
Cytochrome c-catalyzed TMPD peroxidation:
Effect of cytochrome c concentration

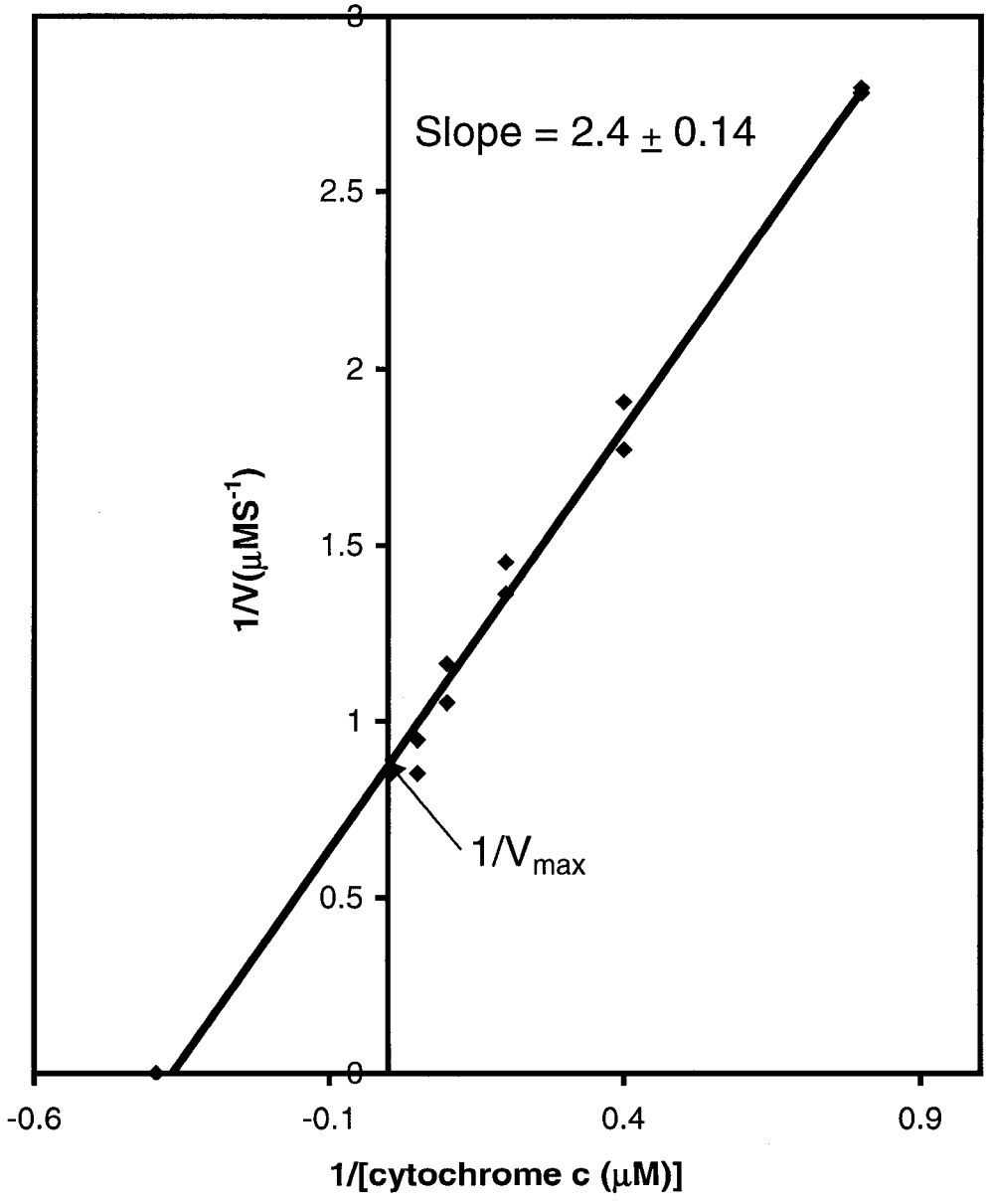


Figure 13. Cytochrome c-catalyzed TMPD peroxidation: Effect of H_2O_2 concentration.

Double-reciprocal plot of reaction velocity as a function of H_2O_2 concentration. 25 µl of cytochrome c stock solution (1.2 mM) was incubated with H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and then buffer and TMPD were added to initiate the reaction. The buffer was 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4). The reactions were conducted at 20°C in a total volume of 3 ml. The final concentrations of TMPD and cytochrome c were 400 µM and 10 µM, respectively. The final concentrations of H_2O_2 were as indicated in the figure. The data plotted were from three different days.

Figure 13
Cytochrome c-catalyzed TMPD peroxidation:
Effect of H_2O_2 concentration

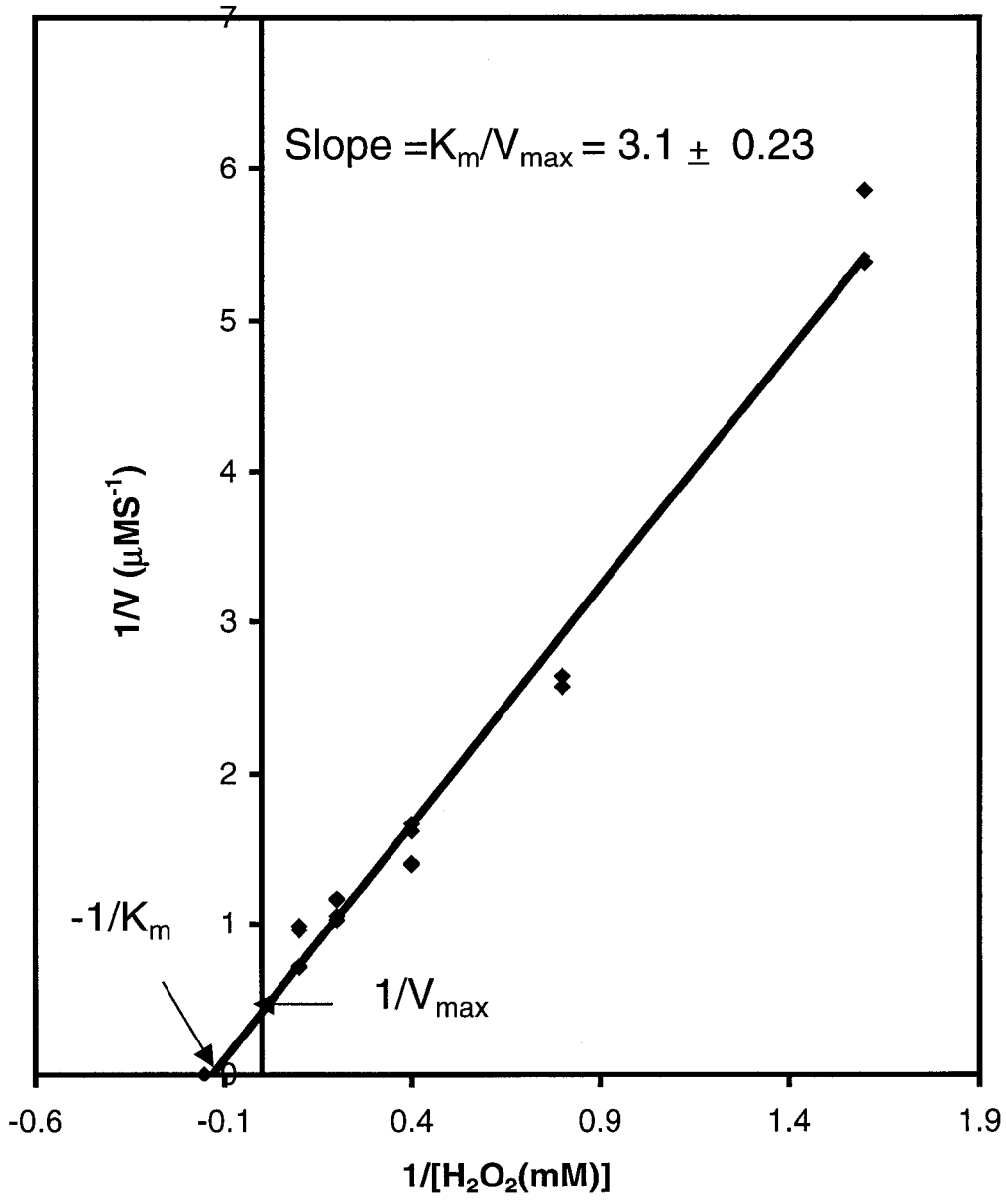


Figure 14. TMPD peroxidation: Effect of temperature.

Logarithm of reaction velocity as a function of reciprocal of absolute temperature. 25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and then buffer and TMPD were added to initiate the reaction. The buffer was 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4). The reactions were conducted at stated temperature in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The figure is representative of reactions run in duplicate on two different days.

Figure 14
TMPD peroxidation:
Effect of temperature

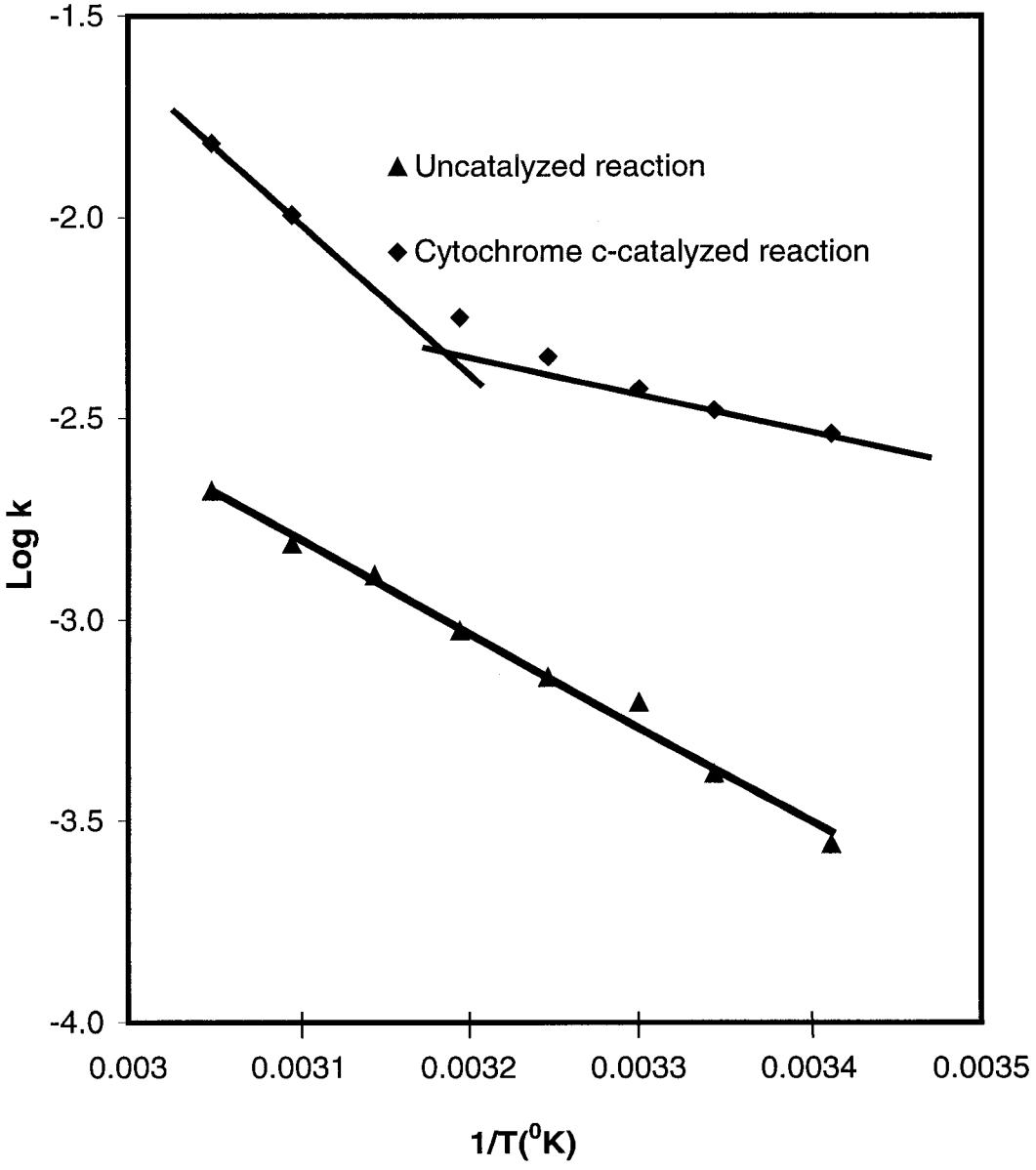


Figure 15. Cytochrome c-catalyzed TMPD peroxidation: Effect of buffer and EDTA.

25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and then stated buffer and 40 µl of TMPD stock solution (30 mM) were added to initiate the reaction. The reactions were conducted at 20°C in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The data plotted were from three different days. Error bars represent standard errors of respective means.

Figure 15
Cytochrome c-catalyzed TMPD peroxidation:
Effect of buffer and EDTA

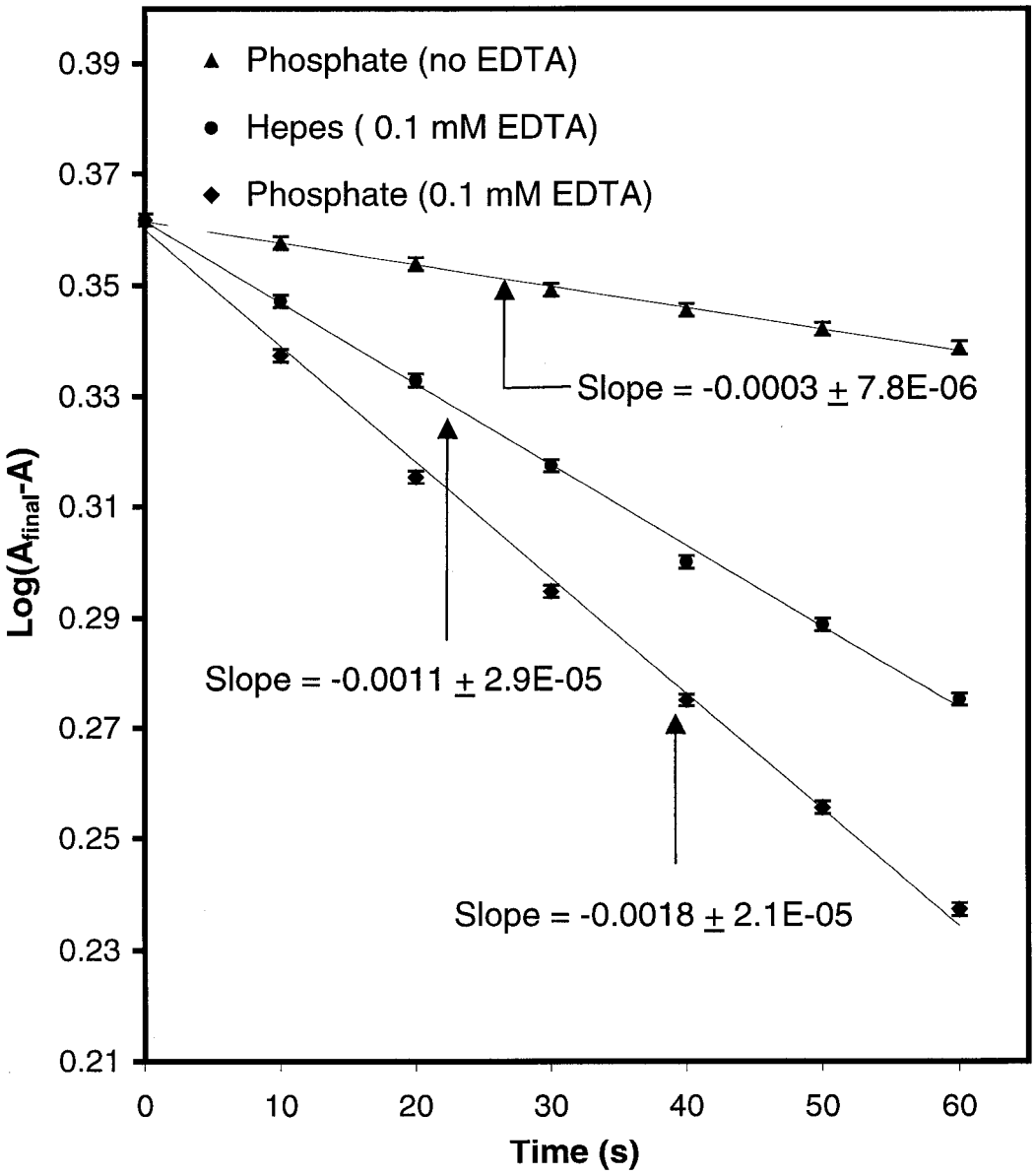
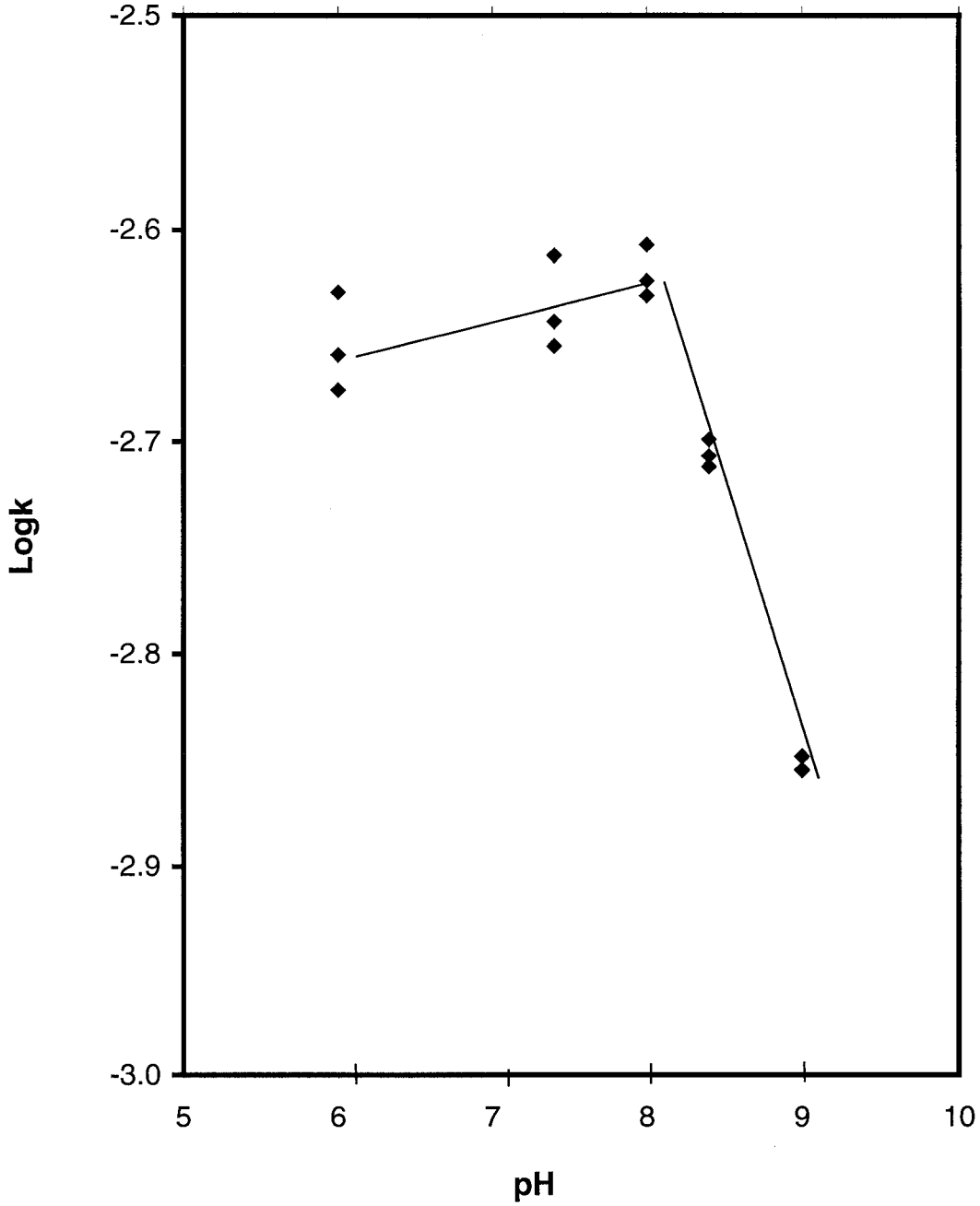


Figure 16. Cytochrome c-catalyzed TMPD peroxidation: Effect of pH.

Logarithm of apparent first order rate constant as a function of pH values of buffer. 25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and then buffer and TMPD were added to initiate the reaction. The buffer was 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4). The reactions were conducted at 20°C in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The data plotted were from three different days.

Figure 16
Cytochrome c-catalyzed TMPD peroxidation:
Effect of pH



Increasing ionic strength decreases peroxidatic activity of cytochrome c

The influence of ionic strength on cytochrome c-catalyzed TMPD peroxidation was studied by conducting the reaction in potassium phosphate buffer at pH 7.4 with different concentrations. The data plotted indicate a linear relationship between the logarithms of the rate constant, k, and the square root of the buffer concentration predicted by Debye-Hückel theory (Figure 17). This straight line with negative slope suggests a substantial decrease of charge in going from the ground state to the transition state.

Halides increase TMPD peroxidation

The halides Cl^- and I^- increased the cytochrome c-catalyzed TMPD peroxidation in a dose dependent manner. Adding metal chelators slowed the reaction, but the catalysis-enhancing effects of Cl^-, and I^- persisted (Figure 18). Compared with Cl^-, the enhancement by I^- was stronger. I^- also strongly increased the uncatalyzed TMPD peroxidation (Figure 19) while Cl^- did not (data not shown). The enhancement by I^- on uncatalyzed TMPD peroxidation was stronger compared with its effect on cytochrome c-catalyzed TMPD peroxidation.

Calcium and zinc inhibit, but copper and iron enhance TMPD peroxidation

The effects of cations on TMPD peroxidation were studied using calcium chloride, zinc sulphate, cupric sulphate, ferrous sulphate, and ferric chloride in potassium phosphate buffer at pH 7.4, in the presence of EDTA.

Ca^{2+} and Zn^{2+} decrease peroxidatic activity of cytochrome c

Calcium had no effect on uncatalyzed TMPD peroxidation, but strongly decreased cytochrome c-catalyzed TMPD peroxidation when its concentration was 75 μM or greater (Figure 20a). For example, adding 125 μM inhibited the cytochrome c-catalyzed TMPD peroxidation by 50%. Calcium had no effect, however, on cytochrome c-catalyzed TMPD peroxidation in the presence of the metal chelating agents (Figure 20b). Like Ca^{2+}, Zn^{2+} at greater than 75 μM inhibited cytochrome c-catalyzed TMPD peroxidation, but had no effect on uncatalyzed TMPD peroxidation (Figure 21).

Cu^{2+} accelerates peroxidatic activity of cytochrome c

When the concentration was 75 μM or greater (but not otherwise), Cu^{2+} accelerated both uncatalyzed and cytochrome c-catalyzed TMPD peroxidation (Figure 22a). In both reactions, acceleration by $CuSO_4$ was dose dependent. For example, when the concentration of $CuSO_4$ was 75 μM, it accelerated the uncatalyzed and cytochrome c-catalyzed TMPD peroxidation by 9-fold and 60%, respectively. At or above 100 μM, Cu^{2+} accelerated the uncatalyzed and cytochrome c-catalyzed TMPD peroxidation by 18-fold and 2-fold, respectively. Desferrioxamine and DTPA completely inhibited acceleration by $CuSO_4$ in both reactions (Figure 22b).

Fe^{2+} and Fe^{3+} accelerate TMPD peroxidation in the absence of cytochrome c

Both Fe^{2+} and Fe^{3+} accelerated uncatalyzed TMPD peroxidation but had little effect on cytochrome c-catalyzed TMPD peroxidation (Figures 23a and 23b). The catalytic effect cytochrome c was approximately twice that of equimolar (10 μM) $FeCl_3$ or $FeSO_4$.

Free radical scavengers and antioxidants inhibit TMPD peroxidation

Formate, mannitol, and benzoate slow TMPD peroxidation.

Formate and mannitol inhibited uncatalyzed TMPD peroxidation by about 20-30% (Figure 24a) and cytochrome c-catalyzed TMPD peroxidation by about 30-40% (Figure 24b). The inhibition was not dose-dependent over the tested concentration range of 10-100 mM. The inhibition by benzoate, however, first increased then decreased as its concentration increased. Specifically, in cytochrome c-catalyzed TMPD peroxidation, inhibition by benzoate increased from 45% to 49% when its concentration increased from 10 to 25 mM, and then dropped to 43% and 31% as its concentration increased to 50 and 100 mM, respectively. In uncatalyzed TMPD peroxidation, inhibition by benzoate increased from 7% to 27% when its concentration increased from 10 to 25 mM, and then dropped to 8% and 2% as its concentration increased to 50 and 100 mM, respectively.

Urate slows cytochrome c-catalyzed TMPD peroxidation

Within the concentration range tested, urate had little effect on uncatalyzed TMPD peroxidation, but slightly decreased cytochrome c-catalyzed TMPD peroxidation (Figure 25a). However, if 1 mM urate was added before pre-incubating cytochrome c with H_2O_2, it strongly inhibited activation of the peroxidatic activity of cytochrome c (Figure 25b).

Figure 17. Cytochrome c-catalyzed TMPD peroxidation: Effect of ionic strength.

Logarithm of apparent first order rate constant as a function of square root of buffer concentration.

25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and then stated concentrations of potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) and 40 µl TMPD stock solution (30 mM) were added to initiate the reaction. The reactions were conducted at 20°C in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The data plotted were from three different days.

Figure 17
Cytochrome c-catalyzed TMPD peroxidation:
Effect of ionic strength

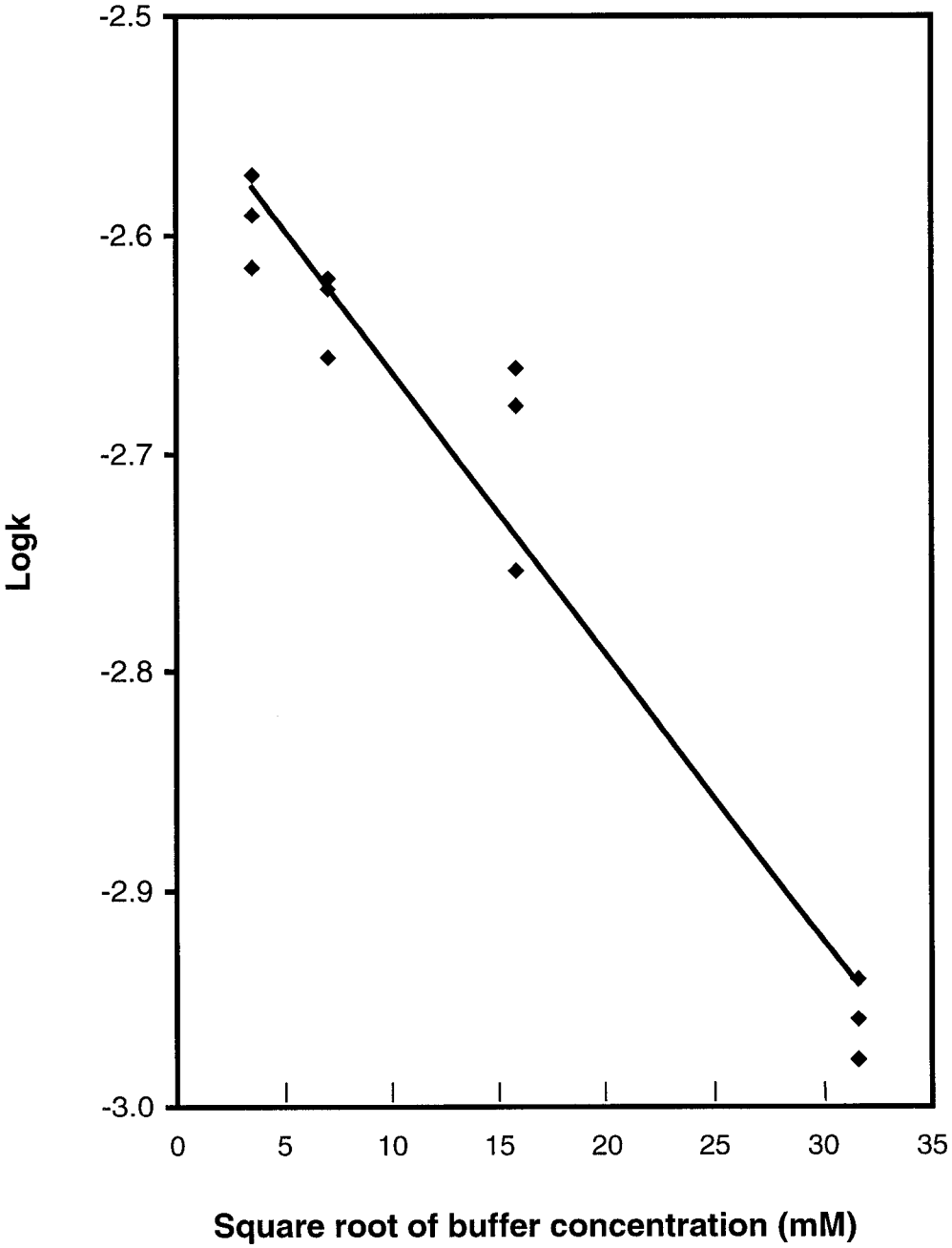


Figure 18. Cytochrome c-catalyzed TMPD peroxidation: Effect of halides.

25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and then buffer and other reagents (except for TMPD) were added to the cuvet. The reactions were initiated by adding TMPD and conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, cytochrome c, and halogen were 400 µM, 5 mM, 10 µM, and 10 mM. The final concentrations of chelators desferrioxamine and DTPA were at 1 mM. The data plotted were from two different days.

Figure 18
Cytochrome c-catalyzed TMPD peroxidation:
Effect of halides

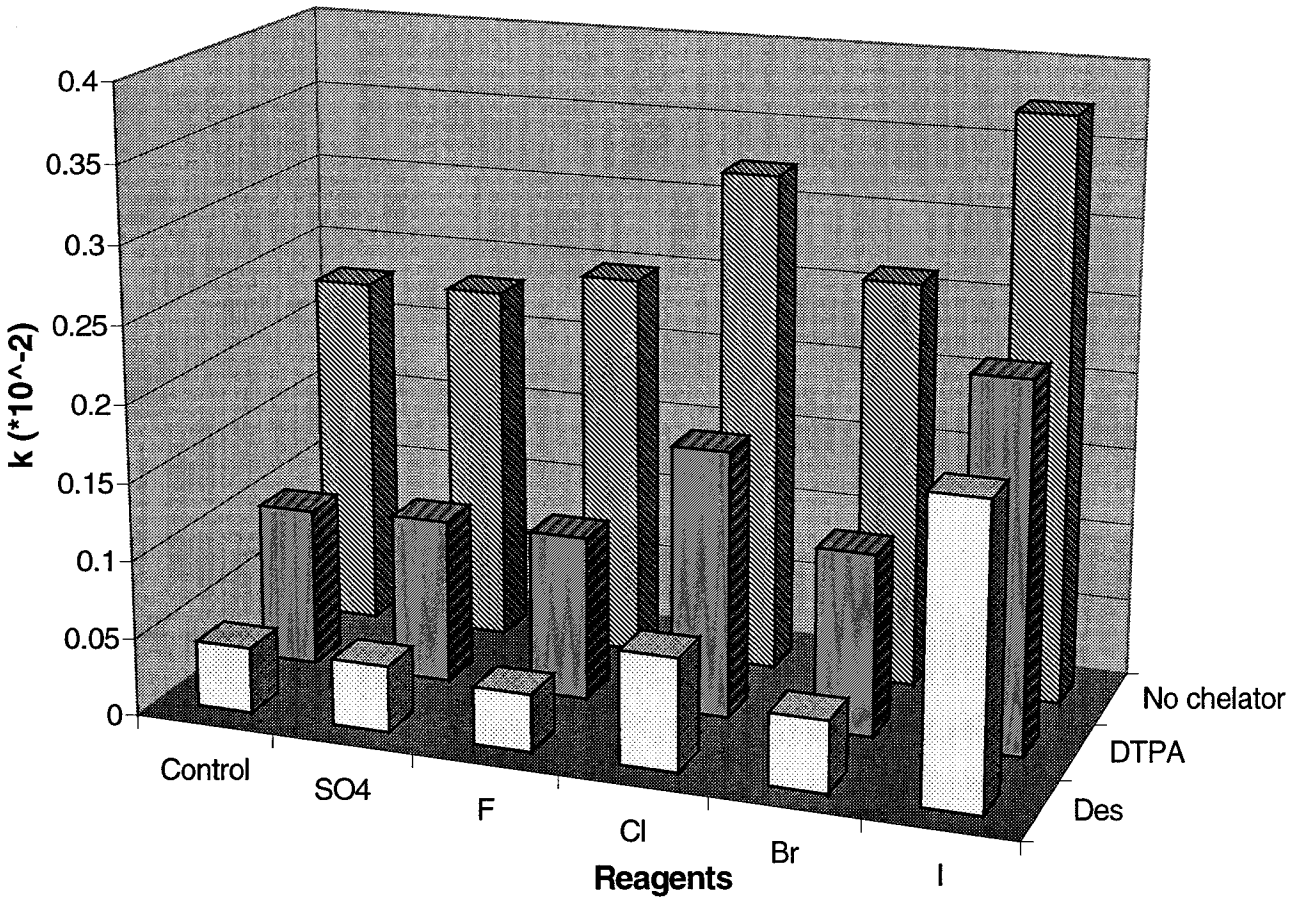


Figure 19. TMPD peroxidation: Effect of NaI.

In cytochrome c-catalyzed TMPD peroxidation, 25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and other reagents (except for TMPD) were added to the cuvet. Then buffer and TMPD were added to initiate the reaction. In uncatalyzed TMPD peroxidation, reagents (except for TMPD) were added to the cuvet, and then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, cytochrome c, halogen, desferrioxamine, and DTPA were 400 µM, 5 mM, 10 µM, 10 mM, 1 mM, and 1 mM, respectively. The data plotted were from two different days.

Figure 19
TMPD peroxidation:
Effect of NaI

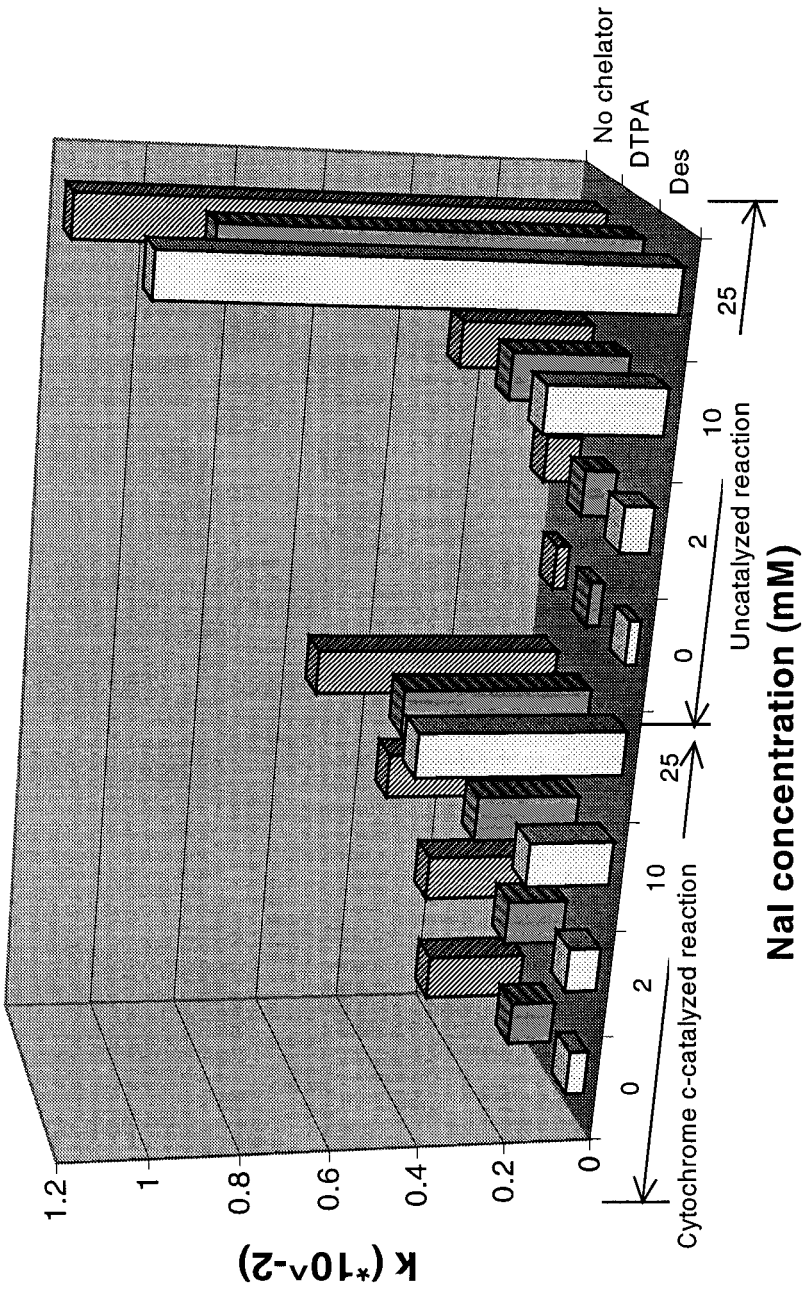


Figure 20 (a). TMPD peroxidation: Effect of $CaCl_2$.

In cytochrome c-catalyzed TMPD peroxidation, 25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and then the stated amount of $CaCl_2$ was added to the cuvet. Then buffer and TMPD were added to initiate the reaction. In uncatalyzed TMPD peroxidation, H_2O_2 and stated amount of $CaCl_2$ were added to the cuvet, and then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The data plotted were from two different days.

Figure 20 (a)
TMPD peroxidation:
Effect of $CaCl_2$

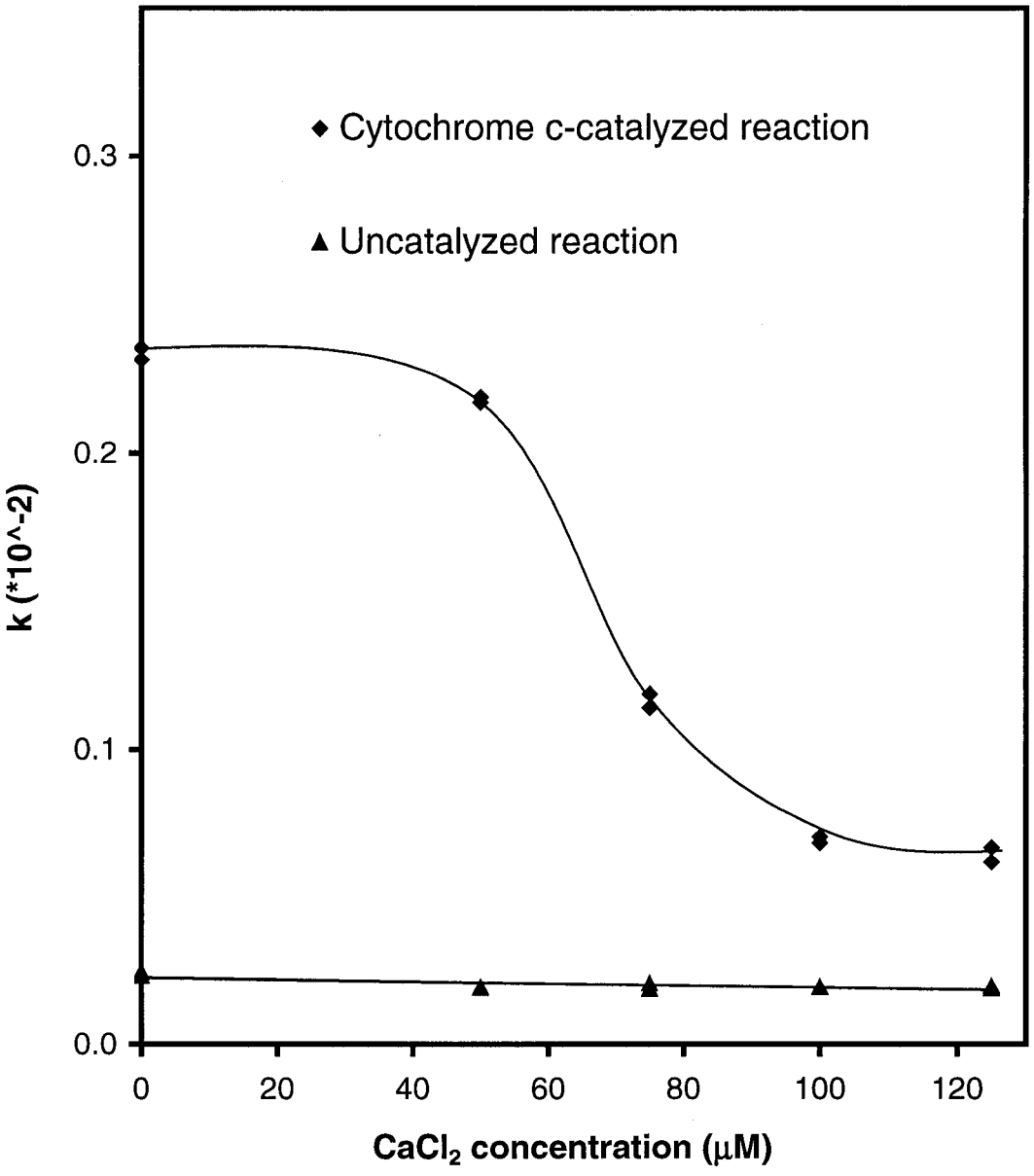


Figure 20 (b). Cytochrome c-catalyzed TMPD peroxidation: Effect of $CaCl_2$ in the presence of metal chelator.

25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and metal chelators and stated amount of $CaCl_2$ were added to the cuvet. Then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, cytochrome c, desferrioxamine, and DTPA were 400 µM, 5 mM, 10 µM, 1 mM, and 1 mM, respectively. The data plotted were from two different days. Error bars represent standard errors of respective means.

Figure 20 (b)
Cytochrome c-catalyzed TMPD peroxidation:
Effect of $CaCl_2$ in the presence of metal chelator

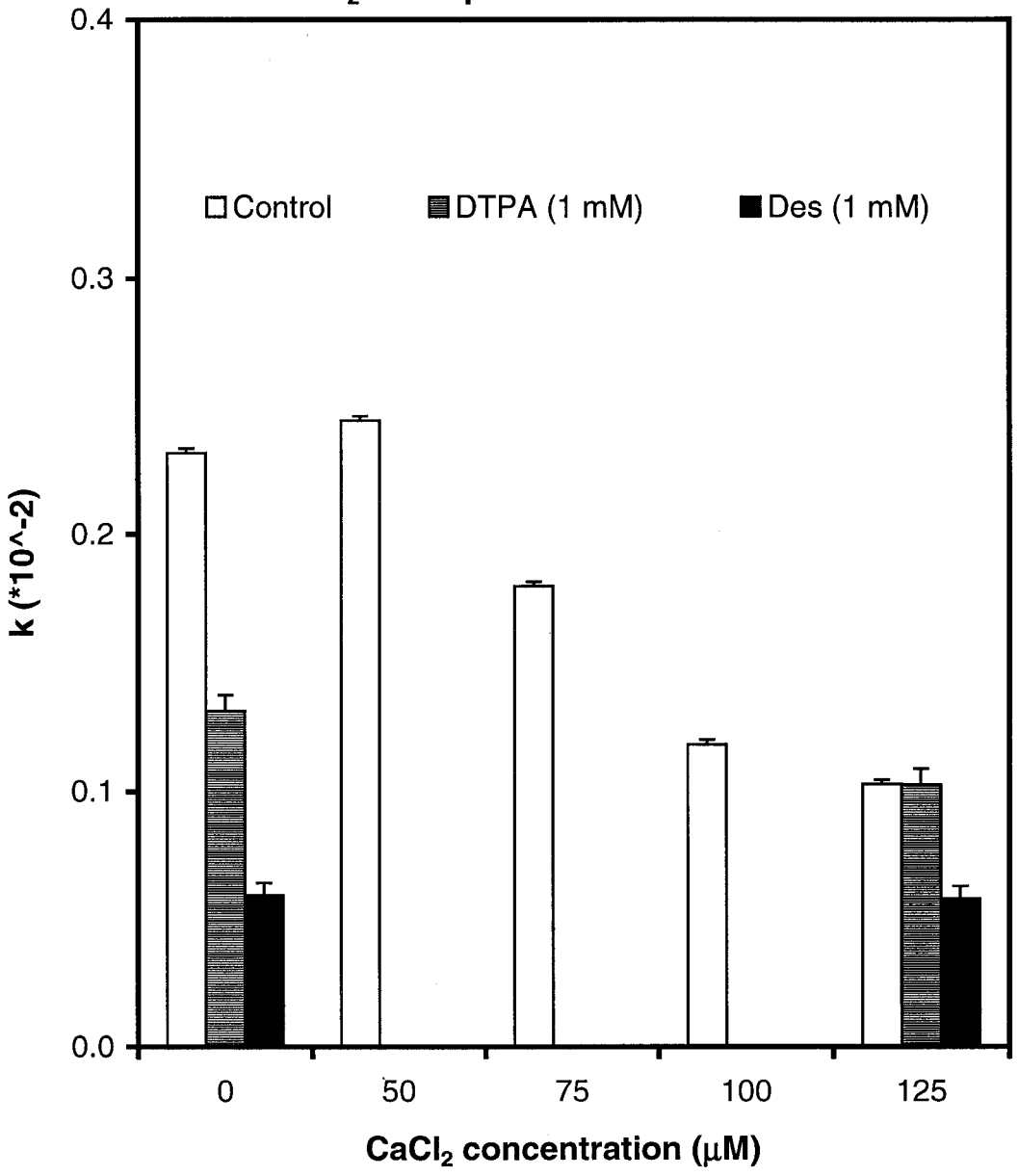


Figure 21. TMPD peroxidation: Effect of $ZnSO_4$.

In cytochrome c-catalyzed TMPD peroxidation, 25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and stated amount of $ZnSO_4$ was added to the cuvet. Then buffer and TMPD were added to initiate the reaction. In uncatalyzed TMPD peroxidation, H_2O_2 and stated amount of $ZnSO_4$ were added to the cuvet, and then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The data plotted were from three different days.

Figure 21
TMPD peroxidation:
Effect of $ZnSO_4$

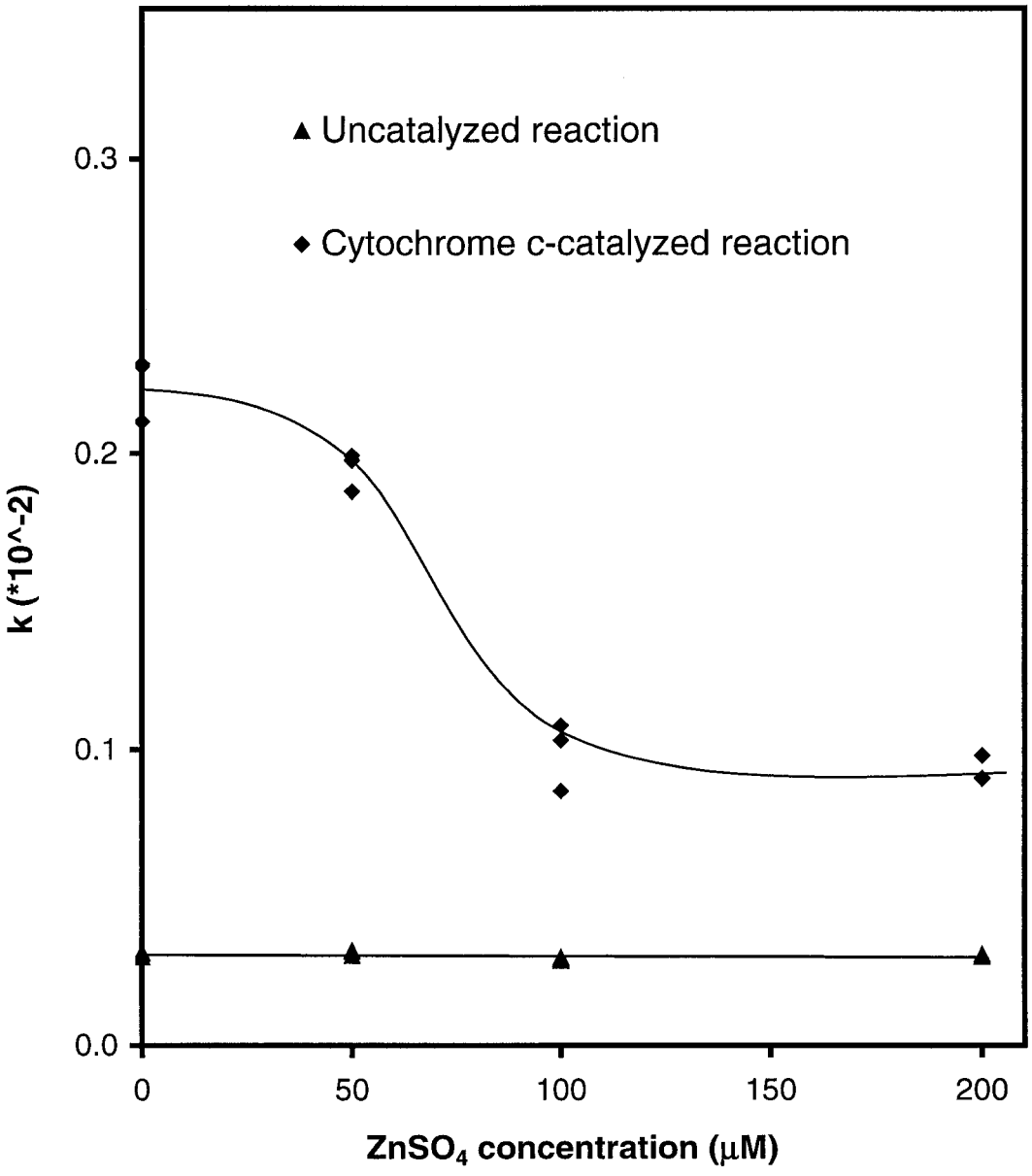


Figure 22 (a). TMPD peroxidation: Effect of $CuSO_4$.

In cytochrome c-catalyzed TMPD peroxidation, 25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and stated amount of $CuSO_4$ was added to the cuvet. Then buffer and TMPD were added to initiate the reaction. In uncatalyzed TMPD peroxidation, H_2O_2 and stated amount of $CuSO_4$ were added to the cuvet, and then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The data plotted were from two different days.

Figure 22 (a)
TMPD peroxidation:
Effect of $CuSO_4$

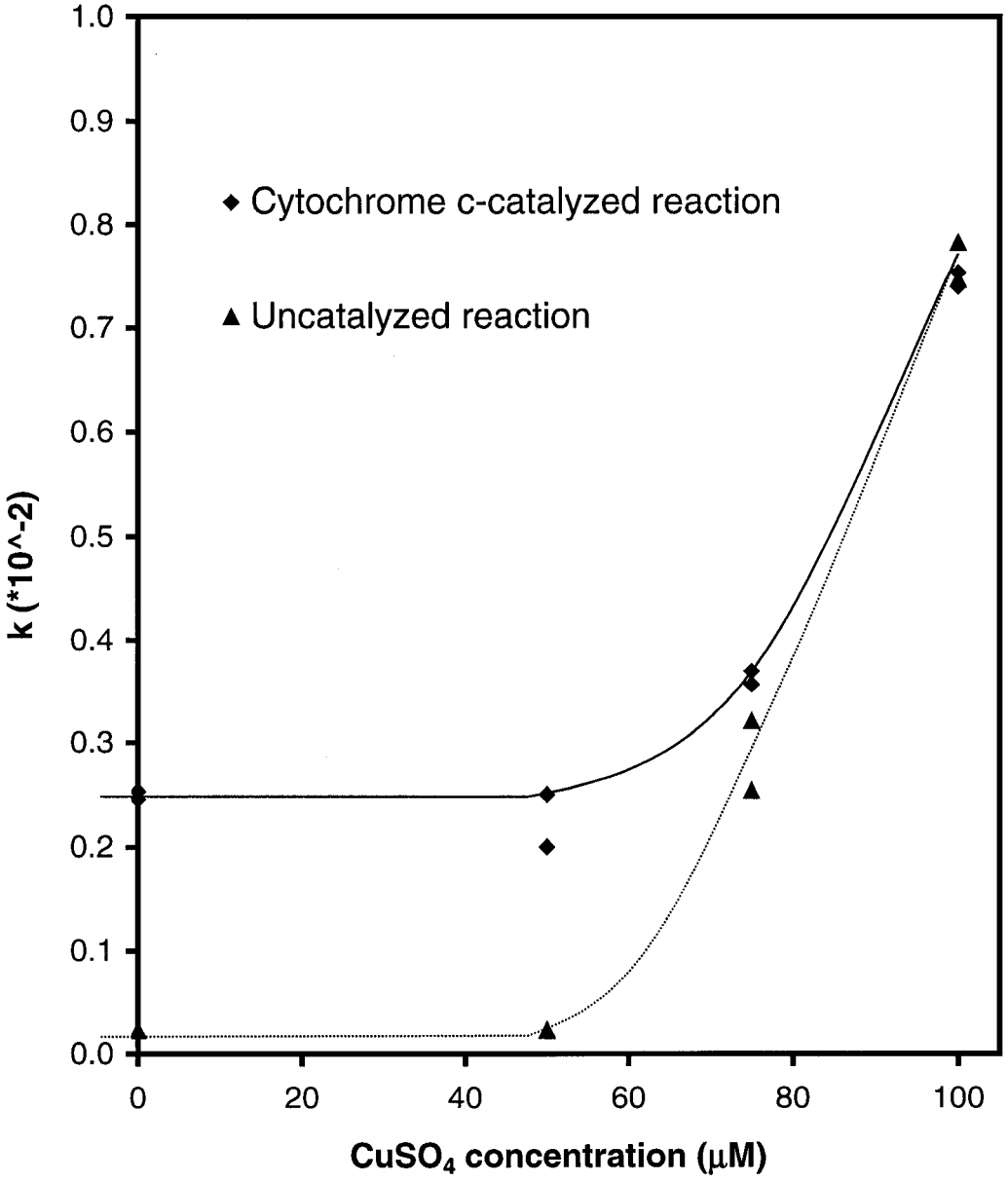


Figure 22 (b). TMPD peroxidation: Effect of $CuSO_4$ in the presence of metal chelator.

In cytochrome c-catalyzed TMPD peroxidation, 25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and metal chelators and stated amount of $CuSO_4$ were added to the cuvet. Then buffer and TMPD were added to initiate the reaction. In uncatalyzed TMPD peroxidation, H_2O_2, metal chelators and stated amount of $CuSO_4$ were added to the cuvet, and then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50°mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, cytochrome c, desferrioxamine, and DTPA were 400 µM, 5 mM, 10 µM, 1 mM, and 1 mM, respectively. The data plotted were from two different days. Error bars represent standard errors of respective means.

Figure 22 (b)
TMPD peroxidation:
Effect of $CuSO_4$ in the presence of metal chelators

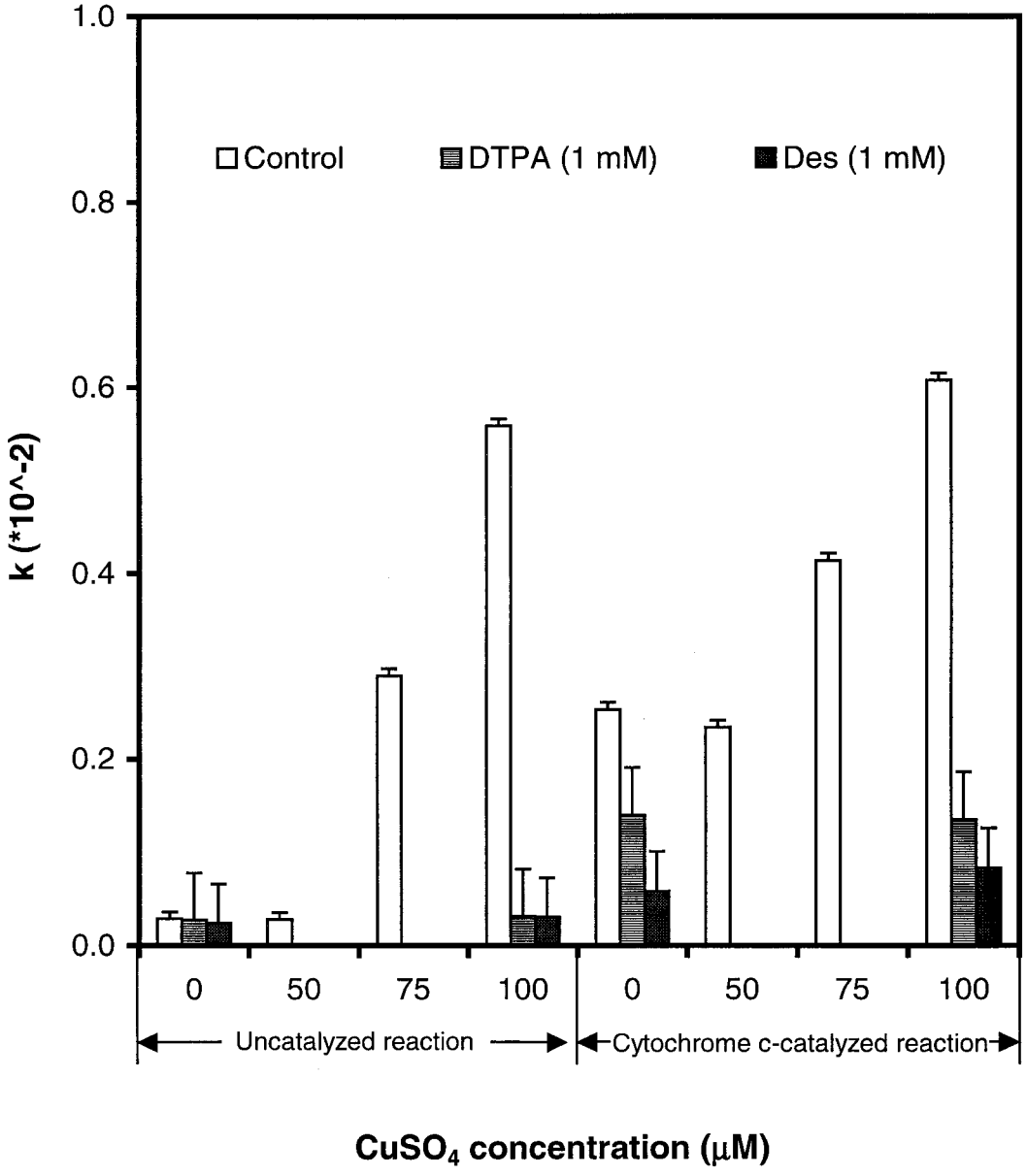


Figure 23 (a). TMPD peroxidation: Effect of Fe^{2+}.

In cytochrome c-catalyzed TMPD peroxidation, 25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and stated amount of $FeSO_4$ was added to the cuvet. Then buffer and TMPD were added to initiate the reaction. In uncatalyzed TMPD peroxidation, H_2O_2 and stated amount of $FeSO_4$ were added to the cuvet, and then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The data plotted were from two different days.

Figure 23 (a)
TMPD peroxidation:
Effect of $FeSO_4$

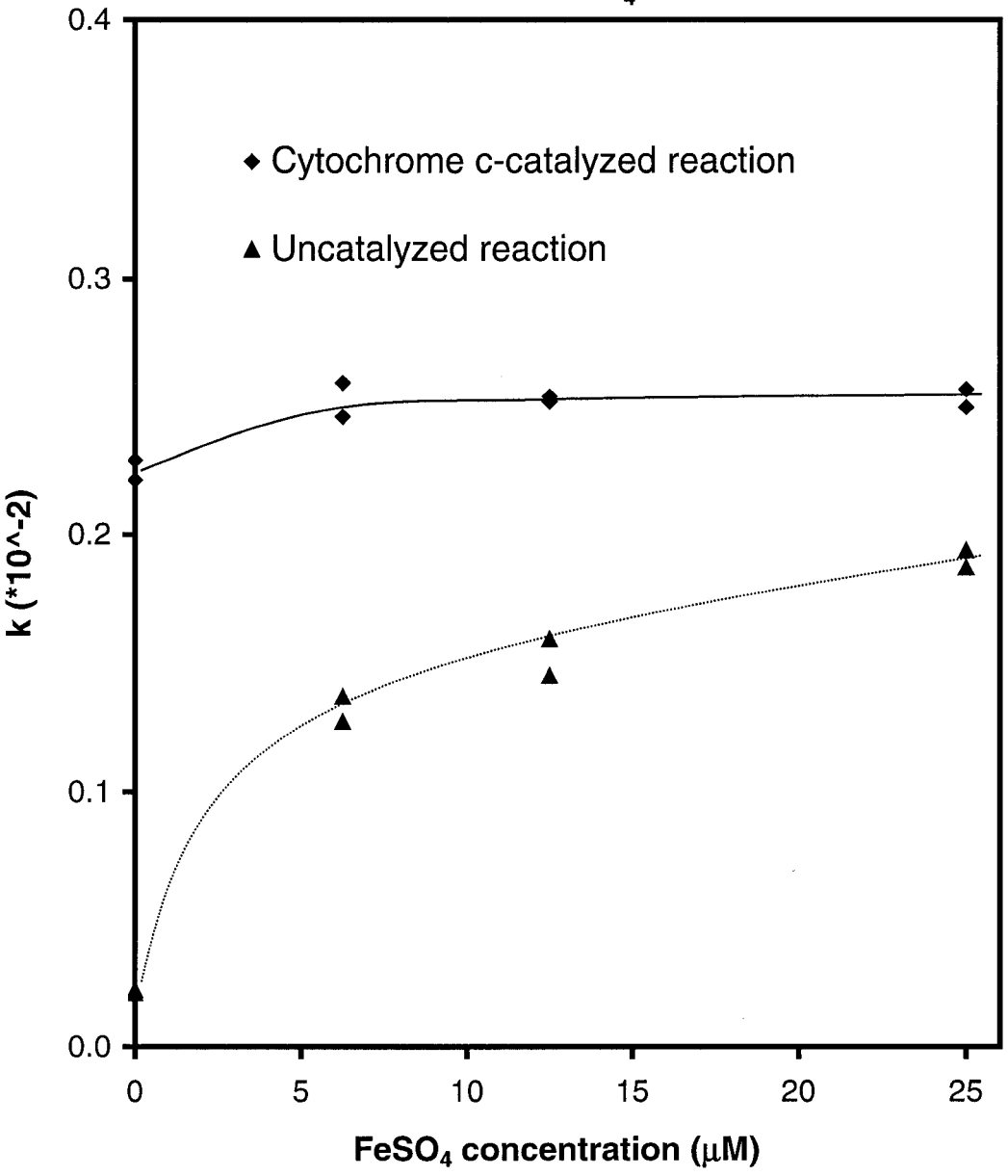


Figure 23 (b). TMPD peroxidation: Effect of Fe^{3+}.

In cytochrome c-catalyzed TMPD peroxidation, 25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and stated amount of $FeCl_3$ was added to the cuvet. Then buffer and TMPD were added to initiate the reaction. In uncatalyzed TMPD peroxidation, H_2O_2 and stated amount of $FeCl_3$ were added to the cuvet, and then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The data plotted were from two different days.

Figure 23 (b)
TMPD peroxidation:
Effect of $FeCl_3$

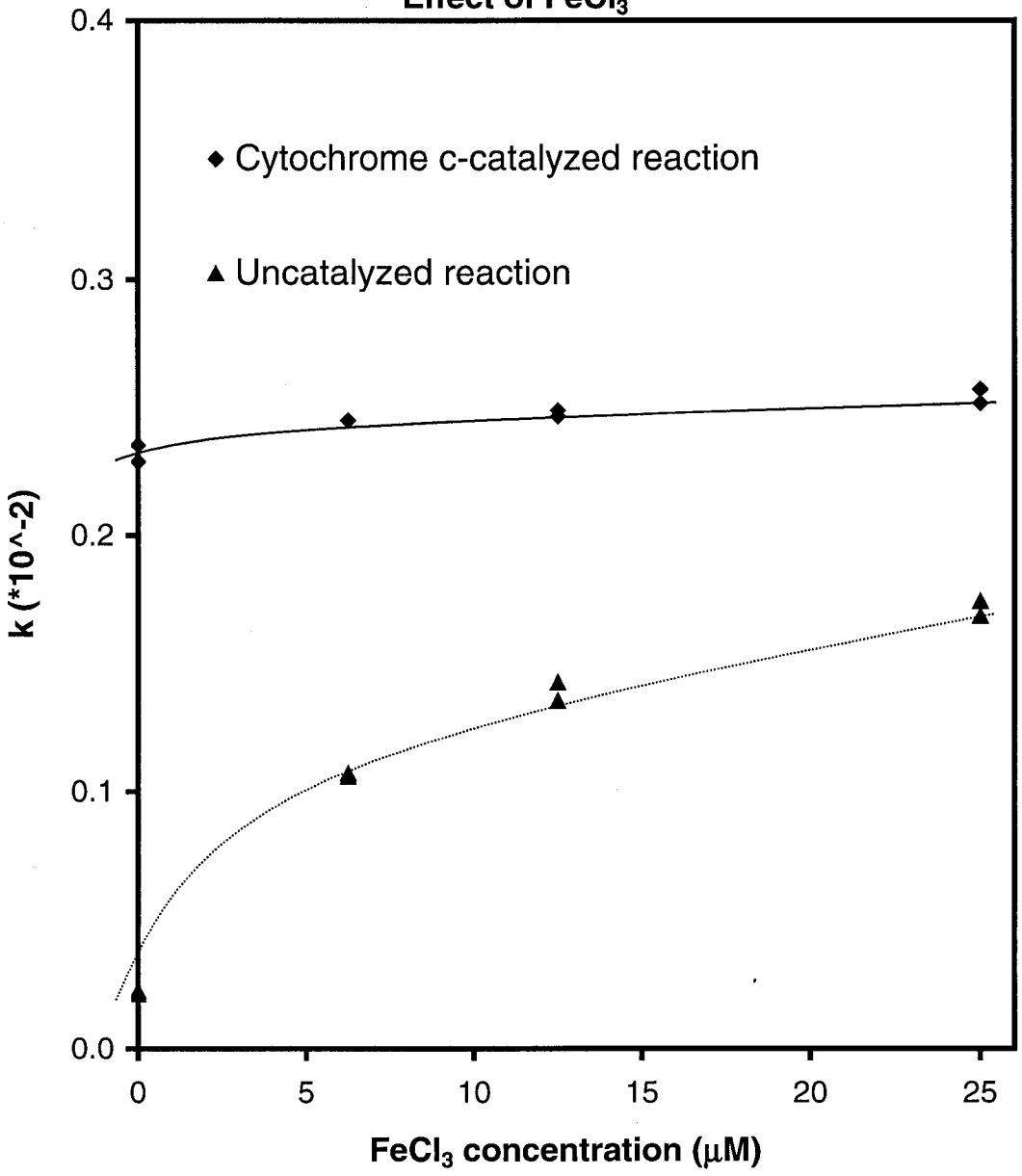


Figure 24 (a). Uncatalyzed TMPD peroxidation: Effect of scavengers.

20 µl of H_2O_2 stock solution (750 mM), plus stated amounts of benzoate, formate, and mannitol were added to the cuvet, and then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD and H_2O_2 were 400 µM and 5 mM, respectively. The data plotted were from two different days.

Figure 24 (a)

Uncatalyzed TMPD peroxidation:
Effect of scavengers

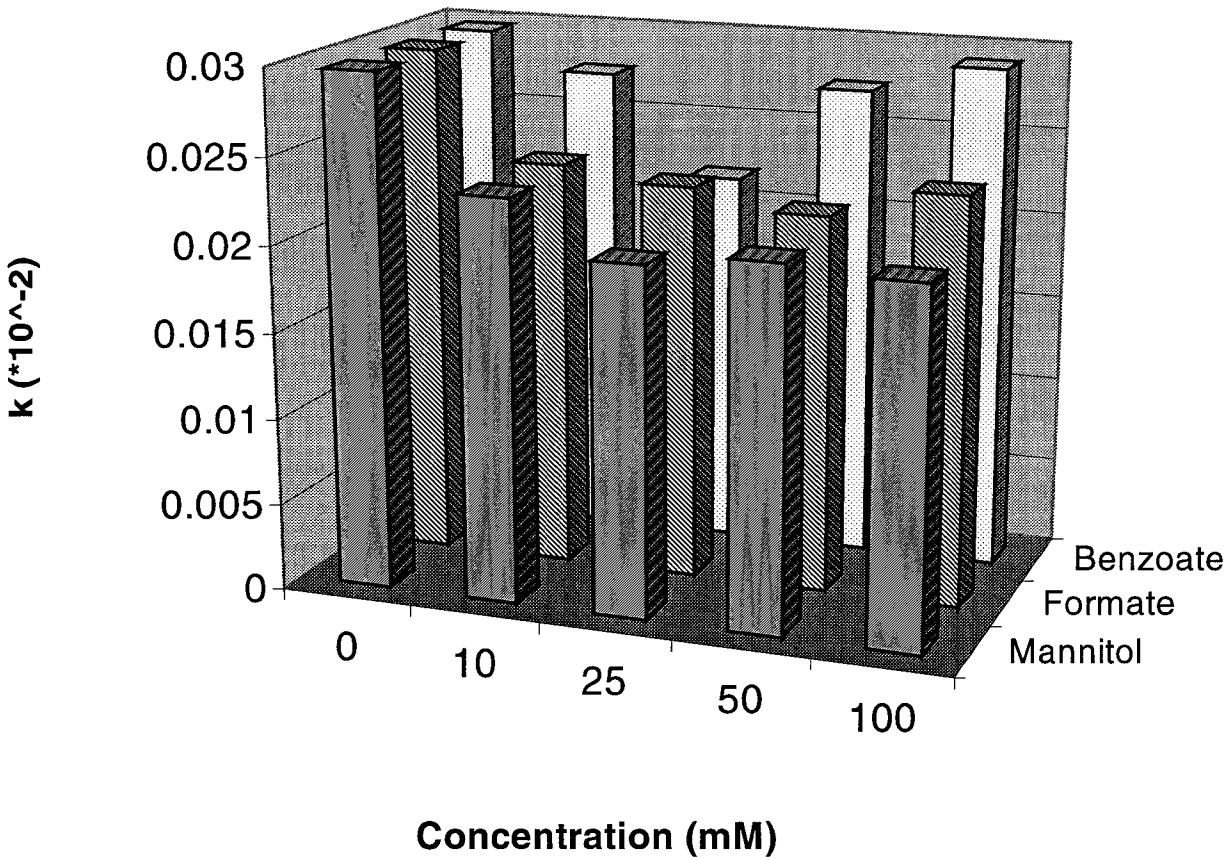


Figure 24 (b). Cytochrome c-catalyzed TMPD peroxidation: Effect of scavengers.

25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and stated amounts of benzoate, formate, and mannitol were added to the cuvet. Then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The data plotted were from two different days.

Figure 24 (b)
Cytochrome c-catalyzed TMPD peroxidation:
Effect of scavengers

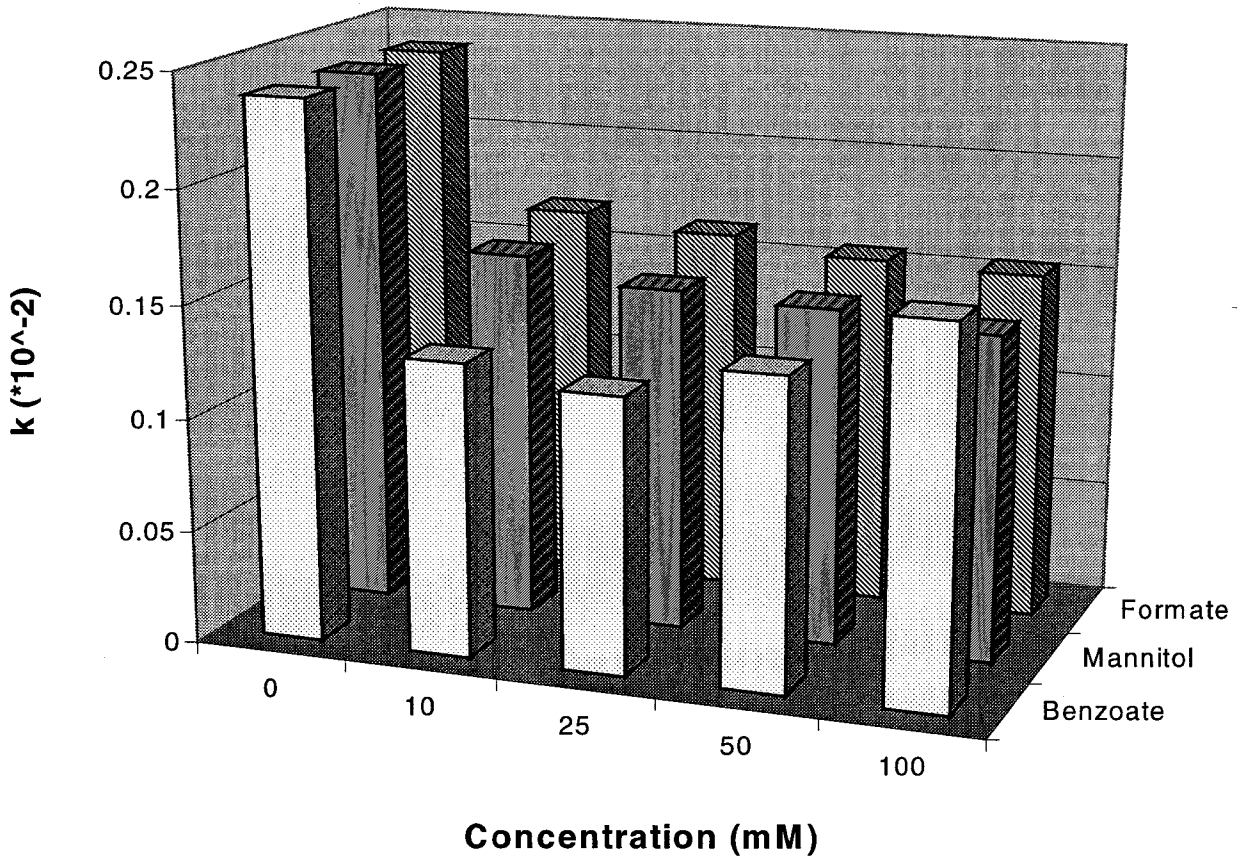


Figure 25 (a). TMPD peroxidation: Effect of urate.

In cytochrome c-catalyzed TMPD peroxidation, 25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and stated amount of urate was added to the cuvet. Then buffer and TMPD were added to initiate the reaction. In uncatalyzed TMPD peroxidation, H_2O_2 and stated amount of urate were added to the cuvet, and then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The data plotted were from two different days.

Figure 25 (a)
TMPD peroxidation:
Effect of urate

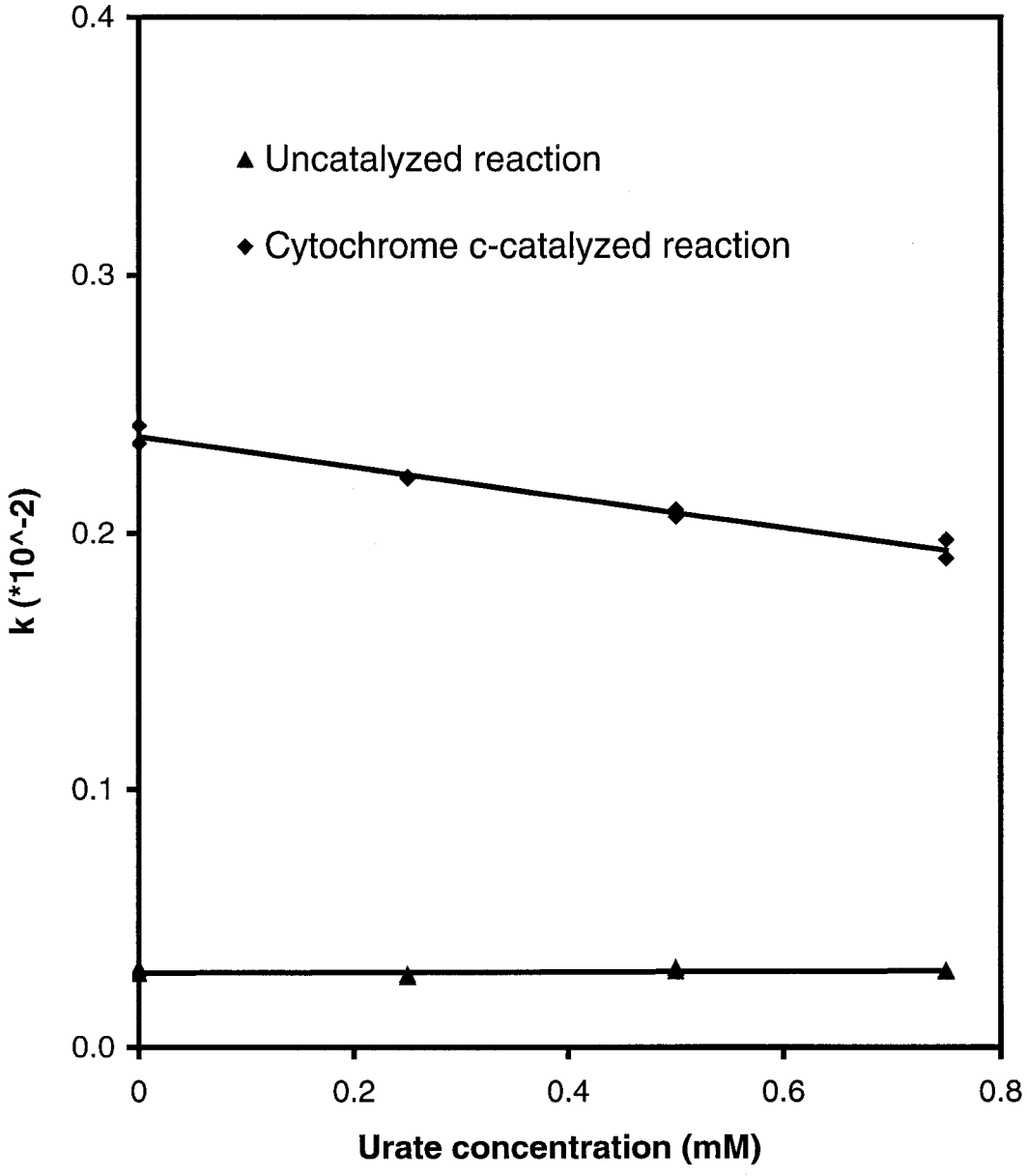
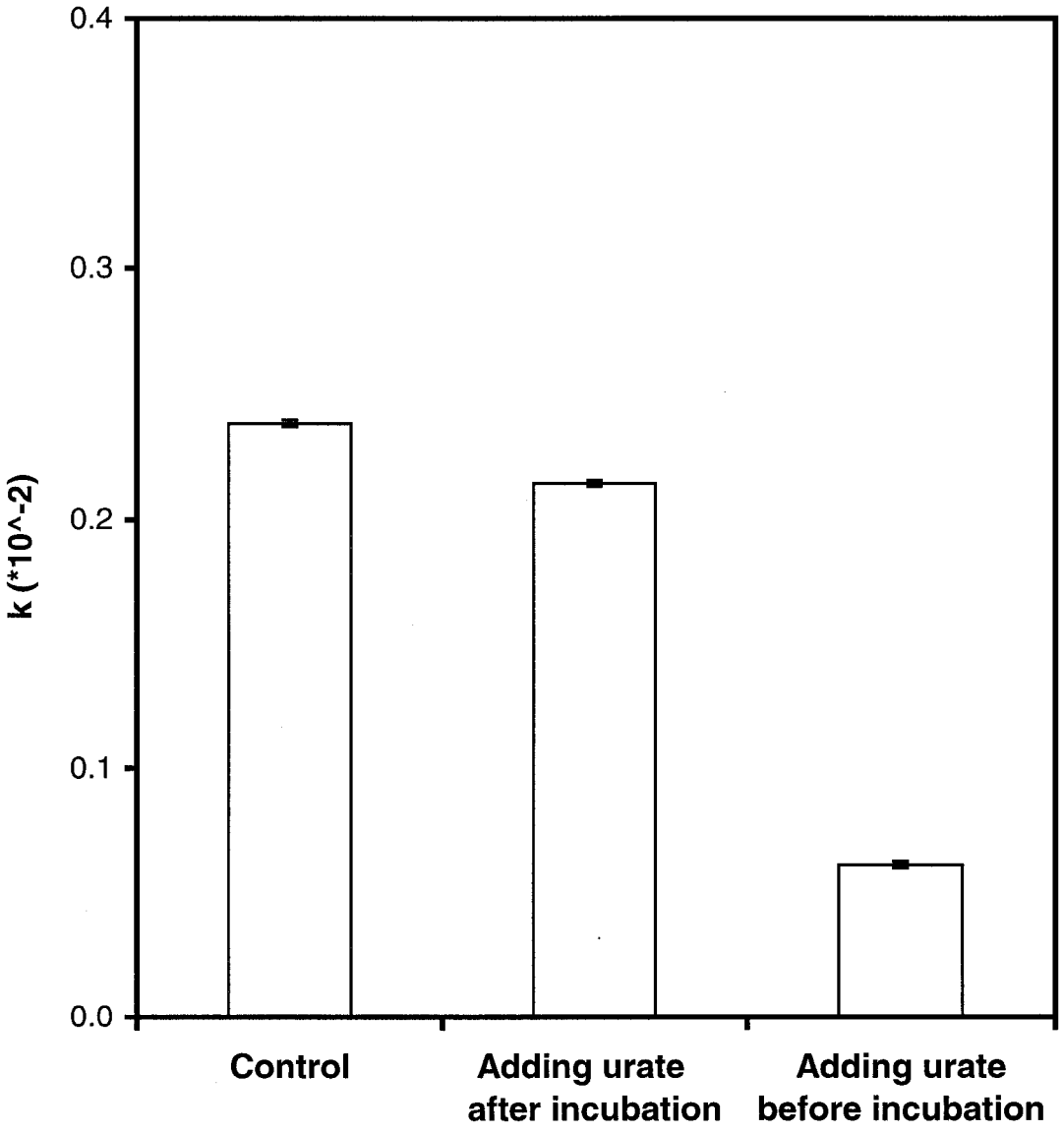


Figure 25 (b). Cytochrome c-catalyzed TMPD peroxidation: Effect of urate with different adding time.

25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and urate was added to the cuvet either before or after the incubation, then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, cytochrome c, and urate were 400 µM, 5 mM, 10 µM, and 1 mM, respectively. The data plotted were from two different days. Error bars represent standard errors of respective means.

Figure 25 (b)
Cytochrome c-catalyzed TMPD peroxidation:
Effect of urate with different addting time.



Ascorbate has dual effects on cytochrome c-catalyzed TMPD peroxidation

The effect of ascorbate on cytochrome c-catalyzed TMPD peroxidation had two stages. In the first stage (latent period), ascorbate completely blocked the reaction (Figure 26a). The duration of the latent period depended on the ascorbate concentration: the higher the ascorbate concentration, the longer the latency. The second stage was acceleration, followed by leveling off.

The concentration with maximum catalytic effect of ascorbate depended on the time of adding ascorbate. If ascorbate was added before incubating cytochrome c with H_2O_2, this concentration was around 100 mM. Otherwise, it was around 25 μM (Figure 26b). The time of adding ascorbate also affected the amplitude of its accelerating effect. Specifically, the accelerating effect was stronger when ascorbate was added after incubating cytochrome c with H_2O_2 compared to that when ascorbate was added before incubating cytochrome c with H_2O_2.

Anthocyanin inhibits peroxidatic activity of cytochrome c

The crude extract of anthocyanins increased uncatalyzed TMPD peroxidation at low concentration (4 μM) but inhibited the reaction by 50% at higher concentration (100 μM or greater). Pure anthocyanins at low concentration (52 μM) had no effect on the uncatalyzed TMPD peroxidation, but inhibited the cytochrome c-catalyzed TMPD peroxidation by up to 85% in a dose dependent manner (Figure 27).

Figure 26 (a). Cytochrome c-catalyzed TMPD peroxidation: Effect of ascorbate.

25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and stated amount of ascorbate was added to the cuvet. Then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The data plotted were from three different days. Error bars represent standard errors of respective means.

Figure 26 (a)
Cytochrome c-catalyzed TMPD peroxidation:
Effect of ascorbate

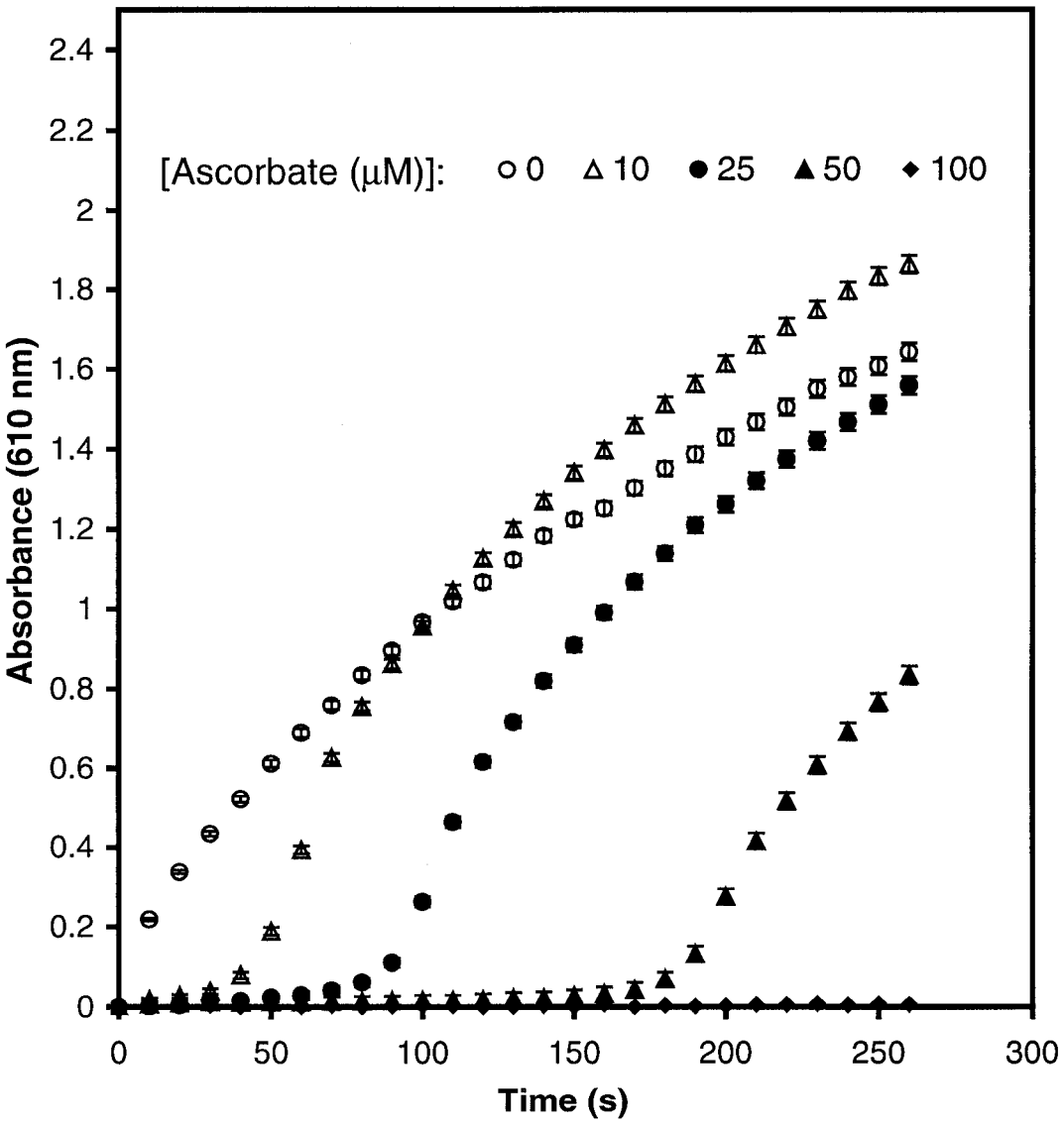


Figure 26 (b). Cytochrome c-catalyzed TMPD peroxidation: Effect of ascorbate with different adding time.

25 μl of cytochrome c stock solution (1.2 mM) was incubated with 20 μl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and stated amount of ascorbate was added to the cuvet either before or after the incubation, then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c, were 400 μM, 5 mM, 10 μM, respectively. The data plotted were from three different days.

Figure 26 (b)
Cytochrome c-catalyzed TMPD peroxidation:
Effect of ascorbate with different adding time

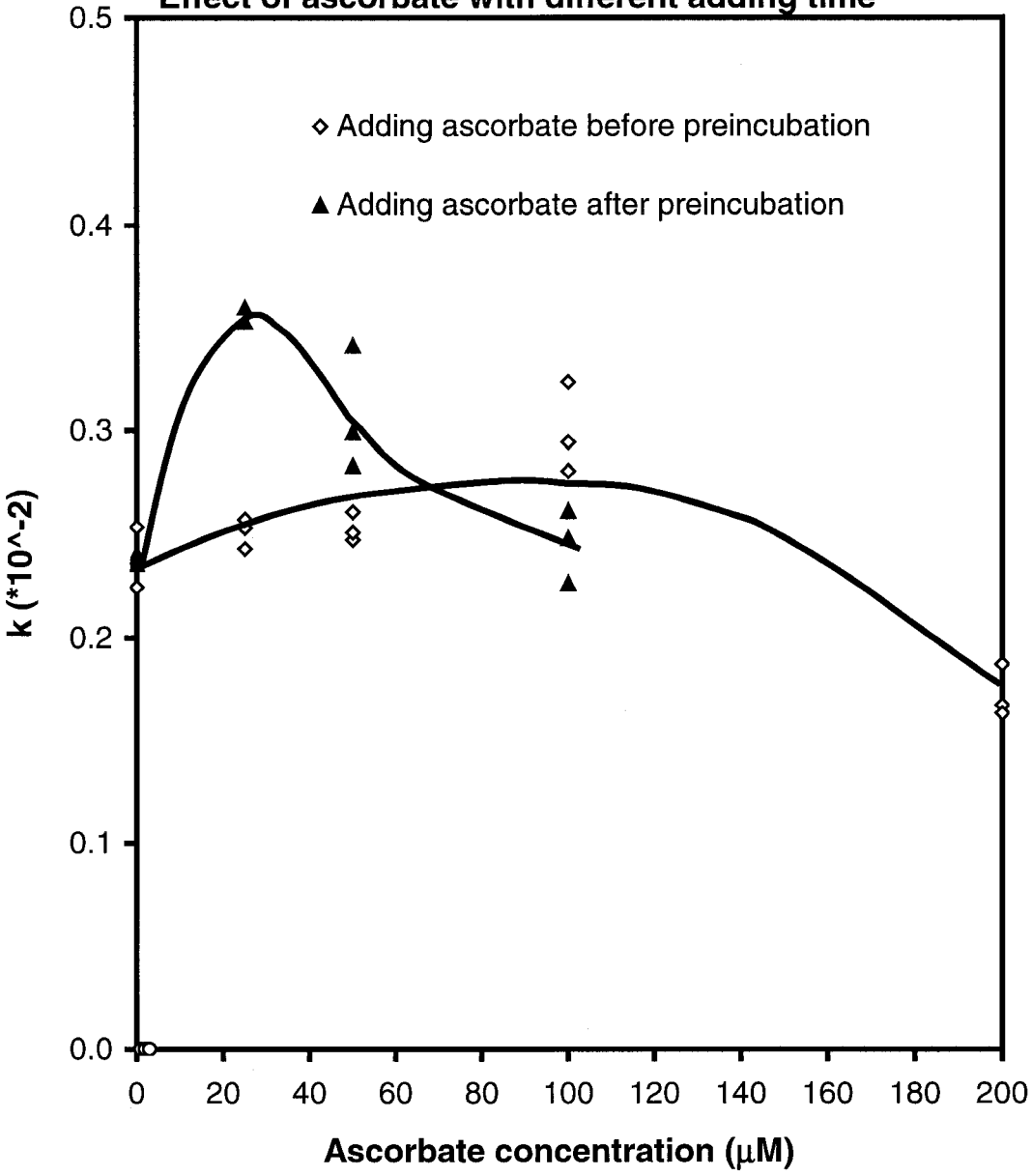
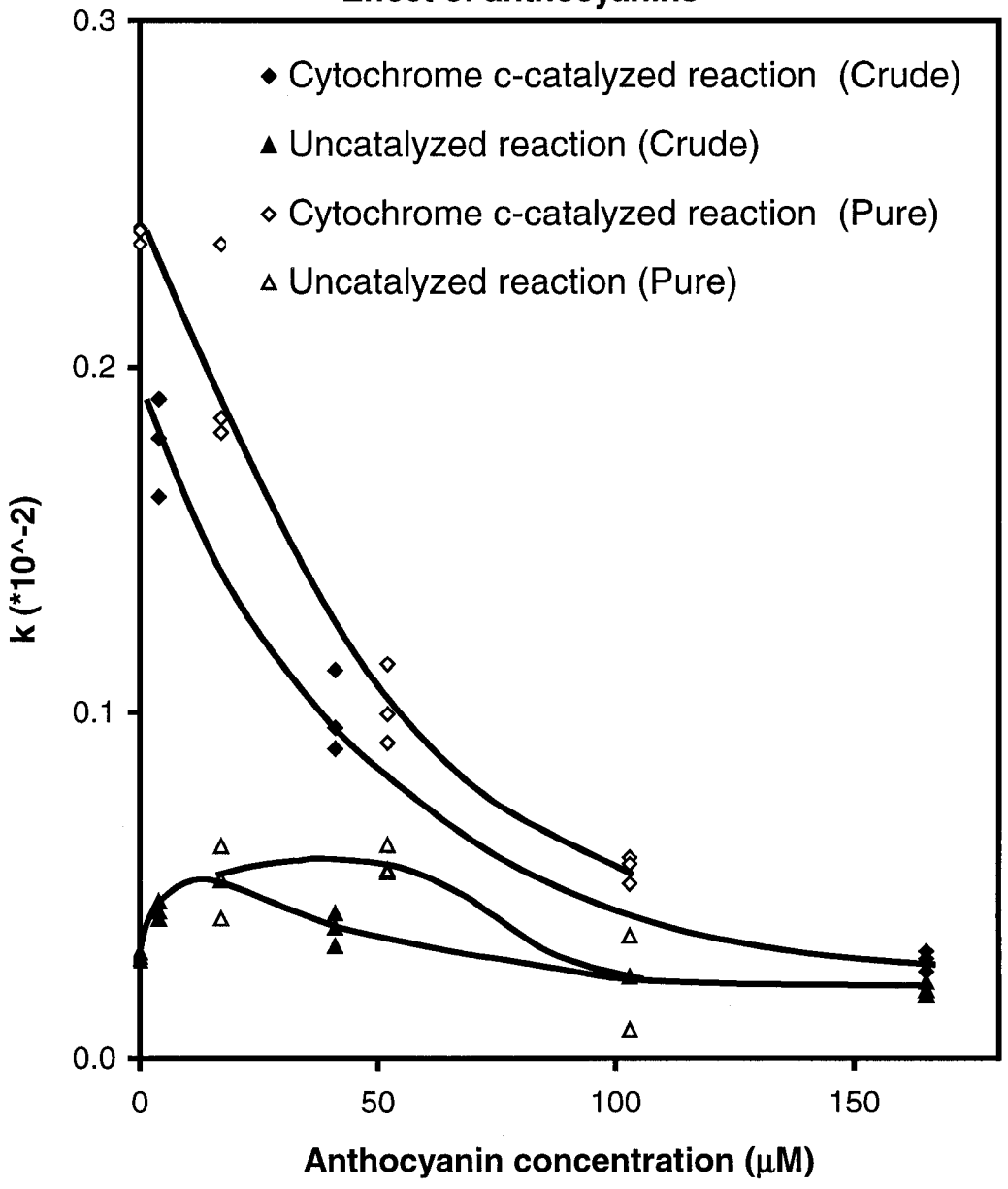


Figure 27. TMPD peroxidation: Effect of anthocyanins.

In cytochrome c-catalyzed TMPD peroxidation, 25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet. Then stated amounts of a crude mixture of anthocyanins and of purified cyanidin-3-O-beta-glucopyranoside were added to the cuvet. Finally, 50 mM potassium phosphate buffer (containing 0.1 mM EDTA, pH 7.4) and TMPD were added to initiate the reaction. In uncatalyzed TMPD peroxidation, H_2O_2 and stated amounts of a crude mixture of anthocyanins and of purified cyanidin-3-O-beta-glucopyranoside were added to the cuvet. Then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The data plotted were from three different days.

Figure 27
TMPD peroxidation:
Effect of anthocyanins



Trolox slightly inhibits peroxidatic activity of cytochrome c

Trolox, a synthetic analogue of alpha-tocopherol but less-hydrophobic, had no effect on uncatalyzed TMPD peroxidation, but mildly inhibited cytochrome c-catalyzed TMPD peroxidation. The inhibition by trolox on the reaction was dose-dependent, but saturated at 20% when trolox concentration reached 2 mM (Figure 28a).

In cytochrome c-catalyzed TMPD peroxidation, adding trolox decreased the final absorbance when compared the point at which all the TMPD was oxidized in Figure 28b vs. Figure 9. Injecting more reduced TMPD after the completion of reaction re-initiated the reaction but adding more cytochrome c or H_2O_2 did not restart the reaction, indicating that trolox incubation might generate a product that could diminish the TMPD radical.

Pre-incubation with H_2O_2 enhances peroxidatic activity of cytochrome c

The catalytic activity of cytochrome c in TMPD peroxidation was modulated by many factors, among which the most striking was a ten-fold increase in catalytic activity when the cytochrome c was pre-incubated with the H_2O_2 for 10 s (Figure 29). During the following 60 min, however, catalytic activity decreased, eventually approaching to a relatively stable level. I selected 10 min as pre-incubation time for most studies since activity then was well above control values, and yet remained relatively stable during the time required to set up the experiments.

Figure 28 (a). TMPD peroxidation: Effect of trolox.

In cytochrome c-catalyzed TMPD peroxidation, 25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and stated amount of trolox (disolved with assitent of alkaline) was added to the cuvet. Then buffer and TMPD were added to initiate the reaction. In uncatalyzed TMPD peroxidation, H_2O_2 and stated amount of trolox were added to the cuvet, and then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The data plotted were from two different days.

Figure 28 (a)
TMPD peroxidation:
Effect of Trolox

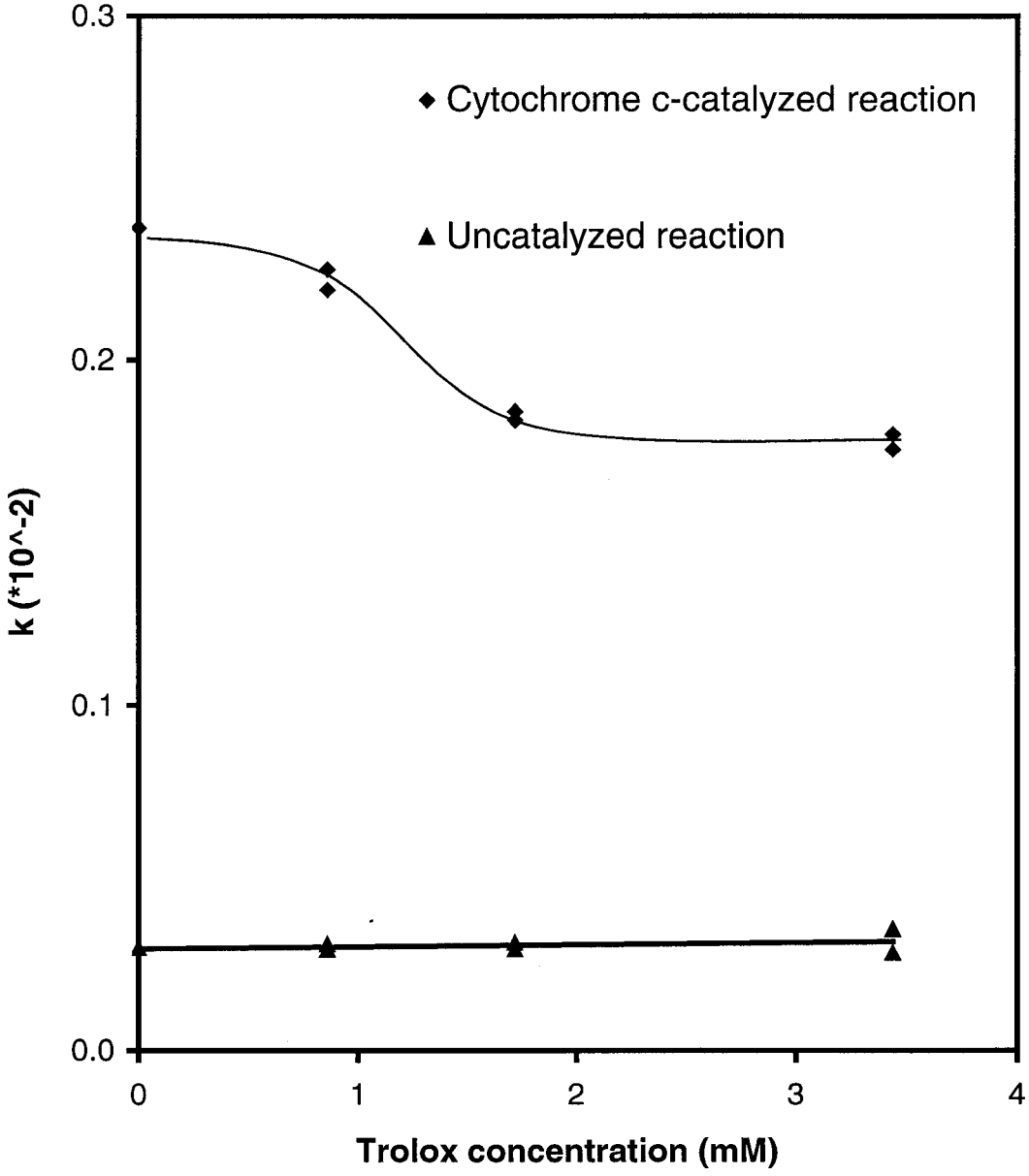


Figure 28 (b). Cytochrome c-catalyzed TMPD peroxidation: Effect of trolox.

25 μl of cytochrome c stock solution (1.2 mM) was incubated with 20 μl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and trolox was added to the cuvet. Then buffer and TMPD were added to initiate the reaction. After the reaction was close to compeletion, stated amounts of TMPD, H_2O_2, and cytochrome c were added to cuvet. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) and total volume of initial reaction mixtures was 3 ml. The final concentrations of TMPD, H_2O_2, cytochrome c, trolox were 400 μM, 5 mM, 10 μM, 3.44 mM, respectively. The data plotted were from two different days.

Figure 28(b)
Catalyzed TMPD peroxidation:
Effect of trolox

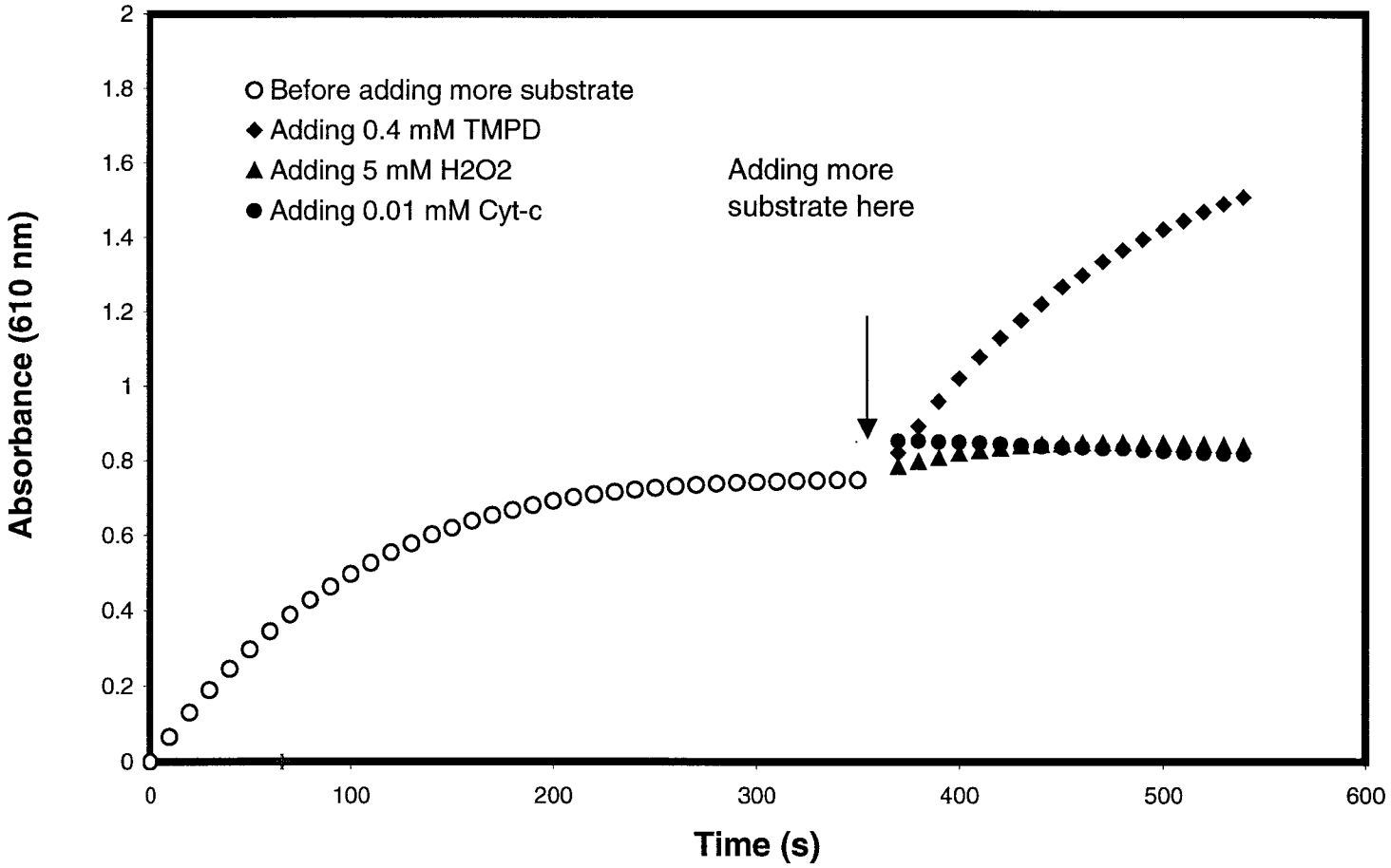
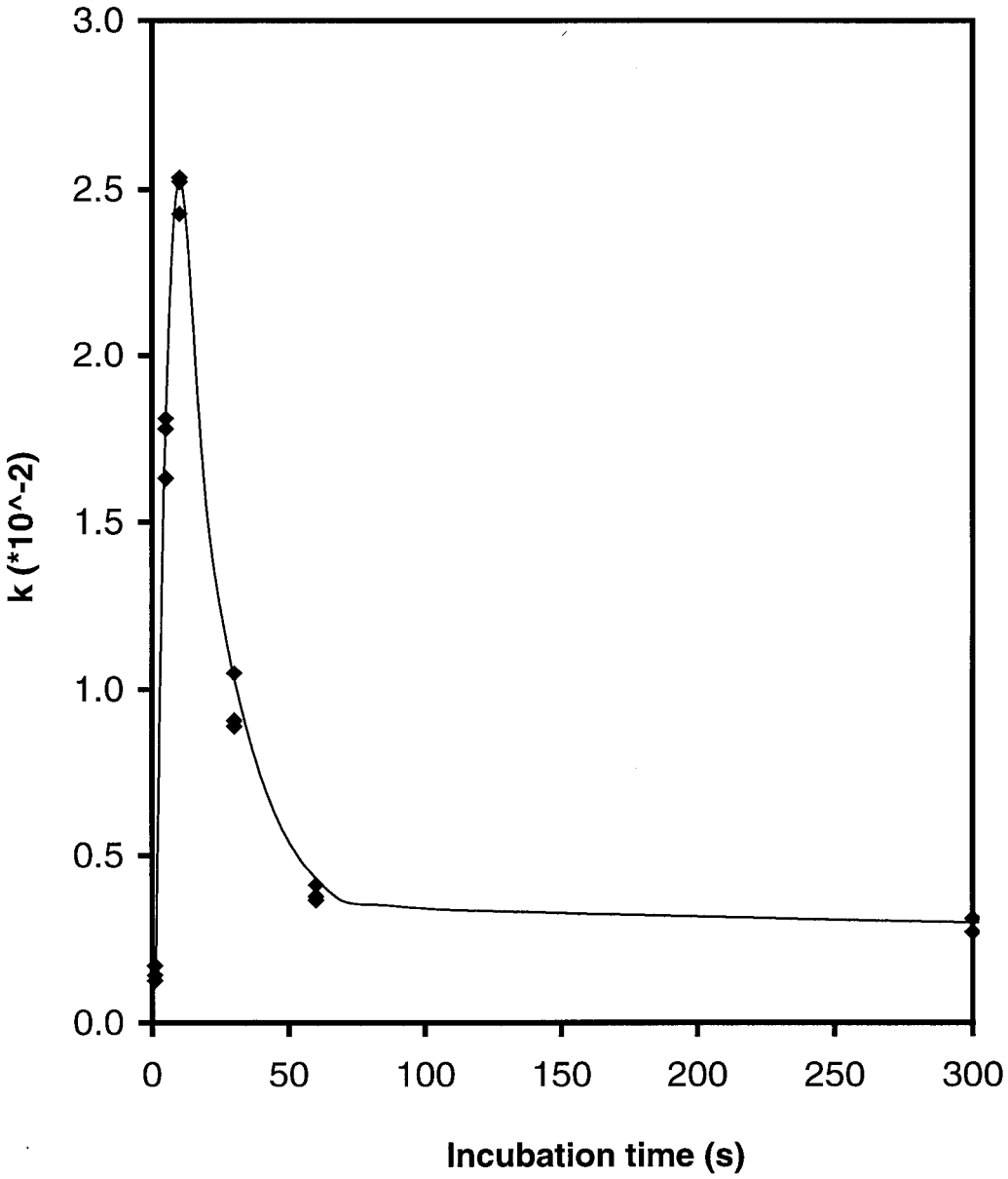


Figure 29. Cytochrome c-catalyzed TMPD peroxidation: Effect of pre-incubation with H_2O_2.

25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for stated amount of time in the cuvet. Then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of TMPD, H_2O_2, and cytochrome c were 400 µM, 5 mM, and 10 µM, respectively. The data plotted were from three different days.

Figure 29
Cytochrome c-catalyzed TMPD peroxidation:
Effect of incubation time



Pre-incubation with H_2O_2 bleaches cytochrome c

In general agreement with earlier results[351] the absorbance of cytochrome c at 411nm (the isosbestic point for the Sorêt peak) decreased by 36.6% after cytochrome c was incubated with H_2O_2 for 10 s. If the incubation time was increased to 10 min, the absorbance decreased 92.5% (Figure 30). When there was no pre-incubation or when cytochrome c was incubated with H_2O_2 for only 10 s and then buffer was added before measurement, the absorbance of cytochrome c at 411 nm continued to decrease. When cytochrome c was incubated with H_2O_2 for 10 min, and then buffer added before the measurement, however, the absorbance of cytochrome c at 411 nm did not change after the initiation of reaction.

Pre-incubation with H_2O_2 causes polymerization of cytochrome c

The results of SDS-PAGE electrophoresis for the effect of pre-incubation of cytochrome c with H_2O_2 show that, instead of being broken into fragments, part of the cytochrome c becomes a dimer or even a trimer (Figure 31, lane 3). Ascorbate seemed to enhance cytochrome c polymerization by H_2O_2 (Figure 31, lane 6 and 7). In contrast, trolox and anthocyanin seemed to inhibit polymerization (Figure 31, lane 4, lane 5, lane 8, and lane 9).

In the following discussion section related to these results, I will first deal with the mechanism of catalysis of cytochrome c on TMPD peroxidation. Then will explain how the reaction condition and factors affect the peroxidatic activity of cytochrome c. Finally,

I will discuss the most important finding of this study: the effect of pre-incubation with H_2O_2 on the peroxidatic activity of cytochrome c.

Figure 30. Bleaching of cytochrome c by H_2O_2.

In reactions without incubation, 25 µl of cytochrome c stock solution (1.2 mM) and 20 µl of H_2O_2 stock solution (750 mM) were added to a cuvet filled with 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4). If the reactions involved incubation, 25 µl of cytochrome c stock solution was incubated with 20 µl of H_2O_2 stock solution for the stated time in the cuvet, and then buffer was added. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH°7.4) in a total volume of 3 ml. The final concentrations of H_2O_2 and cytochrome c were 5 mM and 10 µM, respectively. The data plotted were from three different days. Error bars represent standard errors of respective means.

Figure 30
Bleaching of cytochrome c by H_2O_2

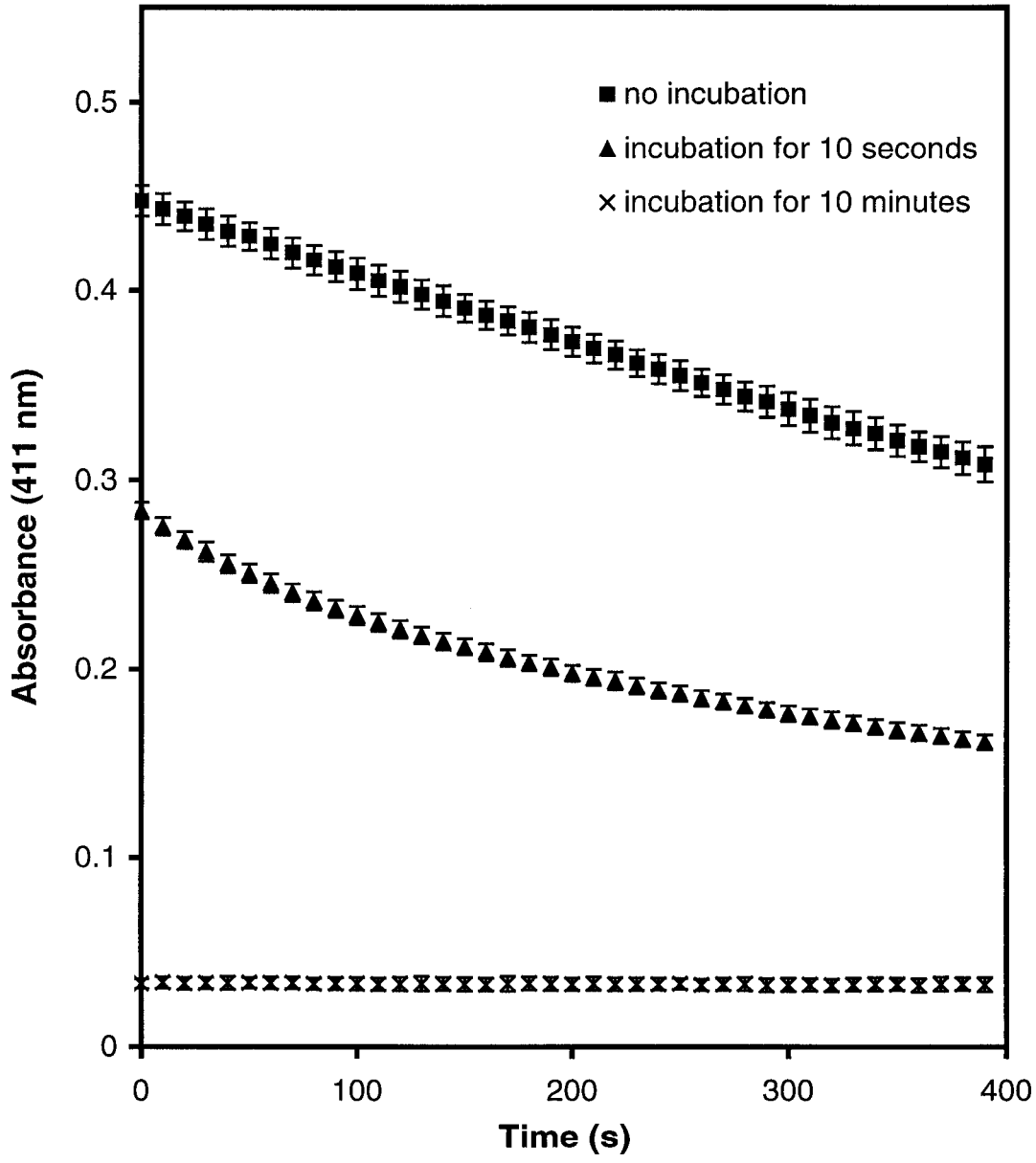


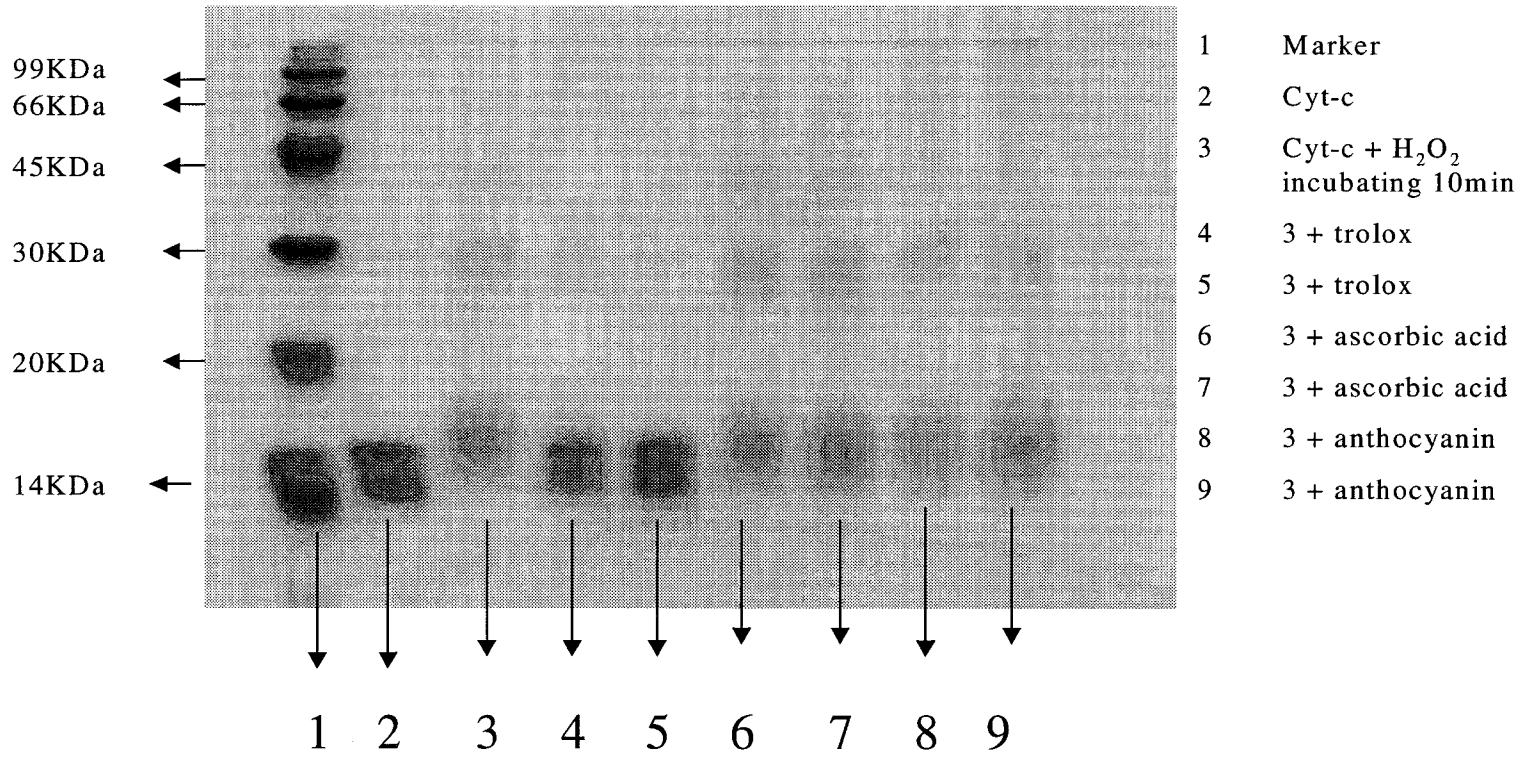
Figure 31. Polymerization of cytochrome c on exposure to H_2O_2.

SDS-PAGE electrophoretogram of the components produced in cytochrome c upon incubation with H_2O_2 under selected reaction conditions.

25 µl of cytochrome c stock solution (1.2 mM) was incubated with 20 µl of H_2O_2 stock solution (750 mM) for 10 min in the cuvet, and other reagents were added to the cuvet. Then buffer and TMPD were added to initiate the reaction. The reactions were conducted at 20°C in 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) in a total volume of 3 ml. The final concentrations of cytochrome c , H_2O_2, trolox, ascorbate, anthocyanin were 10 µM, 5 mM, 1.72 mM, 100 µM , and 165 µM. The figure was representative of reactions run in duplicate on two different days.

Figure 31

Effect of pre-incubation of cytochrome c with H_2O_2



DISCUSSION

Section 1: Peroxidatic activity of superoxide dismutase

Small molecules release peroxidatic activity of Cu,Zn-SOD

The inauguration of peroxidatic activity by bicarbonate (Figure 6), supports previous reports that the Cu,Zn-SOD-H_2O_2 complex releases the free radical generated at its active site in the presence of bicarbonate.[21] Bicarbonate, due to its small size and its negative charge, can get one electron from H_2O_2, pass it to the Cu^{+2} in the active site of Cu,Zn-SOD, and facilitate the oxidation of substrate. Thus, it was not unexpected that Cu,Zn-SOD showed little peroxidatic activity in potassium phosphate buffer at pH 7.4 in the absence of bicarbonate. It was only at unrealistically high concentrations, two orders of magnitude above those necessary to scavenge superoxide, that we observed peroxidatic activity.

Because the pK_a of H_2O_2 is 11.9 and Cu,Zn-SOD binds the hydroperoxy anion, native Cu,Zn-SOD does not display its peroxidatic activity until the pH is raised above 8.[133] In order for Cu,Zn-SOD to exert peroxidatic activity at physiological pH, a small molecule such as bicarbonate is needed to relay the free radical from its active site to react with the substrate, H_2O_2. Otherwise, at neutral pH, H_2O_2 cannot gain access to the positively charged active channel of Cu,Zn-SOD.[21]

In the reaction progress curves in Figure 6, the data in the presence of bicarbonate consist of an initiation phase followed by a faster propagation phase, characteristic of free radical chain reactions. A likely candidate for the propagating species is the carboxylate radical

ion. Observations on the gain of function of superoxide dismutase in the presence of small molecules like bicarbonate are currently receiving increased attention. This is because other workers beside myself have been unable to find widespread peroxidatic activity of either wild-type superoxide dismutase or the mutant forms. Moreover brain carbonyl protein load increases with age, a process which is accelerated in a rodent model for ALS in which mice expressing the lethal mutant superoxide dismutase.[352] With no acceptable hypothesis for the cytotoxic mechanism in ALS, researchers are broadening the search criteria and are now looking at possibilities for small molecules and zinc-depleted superoxide dismutase in the etiology of ALS.[353] With evidence for nitrosylation in post mortem brain from patients with neurodegenerative disorders, there is another candidate besides bicarbonate for the status of the small molecule electron ferry, namely nitric oxide.[354]

Zn-depleted Cu,Zn-SOD has significant peroxidatic activity without requiring the presence of small molecules

This consequence of zinc removal suggests a change in the configuration of superoxide dismutase to allow H_2O_2 (and potentially other abnormal substrates like peroxynitrite) direct access to the active center. Despite speculation as to what the gain of function in the zinc-depleted enzyme might be, mine will be the first report of enhanced peroxidatic activity. This, then, is the significance of my finding that zinc removal conferred enhanced peroxidatic activity in the absence of bicarbonate. This peroxidatic activity was further stimulated by the addition of bicarbonate (Figure 6).

This finding is of interest in relation to the decreased affinity for zinc in the familial amyotrophic lateral sclerosis-associated mutant forms of Cu,Zn-SOD.[22] The results in rodent studies are complicated, but consistent. They are generally consistent with my findings. Thus, mice expressing wild type Cu,Zn-SOD or mutant Zn weakened

superoxide dismutase induce apoptosis in cultured motor neurons, but only if the cultures are zinc depleted. In zinc-adequate cultures, wild type and mutant superoxide dismutase protect agains apoptosis. These data are taken to mean that mutant superoxide dismutase can be functional and protective if it is Zn adequate. They also suggests that wild type superoxide dismutase can be damaging in a zinc deficient animal. It is suggested that neurofilament L may compete with superoxide dismutase for available zinc, leading to loss of zinc even from normal superoxide dismutase (as a possible mechansism for sporadic ALS, but especially in zinc-weakened mutant superoxide dismutase.[353]

The unifying hypothesis then, in both mutant and wild-type superoxide dismutase, in both sporadic ALS and familial ALS, is that removing zinc from Cu,Zn-SOD may render the active site abnormally accessible to redox agents and peroxynitrite,[22] thus permitting a toxic gain-of-function. It also makes the enzyme more vulnerable to damage by reactive oxygen species including H_2O_2,[19] thus allowing weakening of zinc binding in superoxide dismutase and the possibility of a positive feedback loop capable of releasing a pathogenic vicious cycle.

Zn-depleted Cu-SOD is a potential tool for studies of amyotrophic lateral sclerosis

The familial amyotrophic lateral sclerosis-associated mutation of Cu,Zn-SOD at position 124 interferes with the binding domain of zinc, and might well have structural properties similar to the Zn-depleted wild-type derivative obtained by chemical manipulation.[355,356] The Zn-depleted Cu-SOD used in the current studies may mimic the toxic gain-of-

function of those mutants of Cu,Zn-SOD in which Zn-binding is weakened. A range of transgenic rodent models expressing various mutant superoxide dismutase species are available as tools for examining the pathology of ALS in experimental animals.[357]

Small changes in the peroxidatic activity of superoxide dismutase can have significant consequences

The increased peroxidatic activity of Zn-depleted Cu,Zn-SOD is consistent with the superoxide-dependent peroxidase activity of the familial amyotrophic lateral sclerosis-associated Cu,Zn-SOD mutant H48Q studied by Liochev and colleagues.[122] The Zn-binding site of H48Q is also reportedly impaired.[358]

To date, very few of the familial amyotrophic lateral sclerosis-associated mutant Cu,Zn-SOD tested have shown biologically significant increased peroxidatic activity. This therefore runs contrary to the hypothesis that an increased peroxidatic activity of the familial amyotrophic lateral sclerosis-associated mutant Cu,Zn-SOD is the toxic gain-of-function.[13,14,15] Moreover, any acceptable mechanism for amyotrophic lateral sclerosis must also explain why the mutant enzyme remains latent until the onset of disease, and then suddenly releases symptoms in a full scale (possibly through a positive feedback mechanism) required to explain the rapid progress of neuronal death after initiation.

One possible explanation is that even a small "gain-of-function" can decrease the threshold for the switch from the latent to the active stage of the disease. In sporadic amyotrophic lateral sclerosis, such a switch must operate, even in the presence of normal Cu,Zn-SOD. Existing data suggest that the switch that triggers amyotrophic lateral

sclerosis will more likely be found somewhere other than in the modified superoxide dismutases. The timing of onset, and the speed of progression may be modulated by relatively small changes in the native or mutant enzyme.

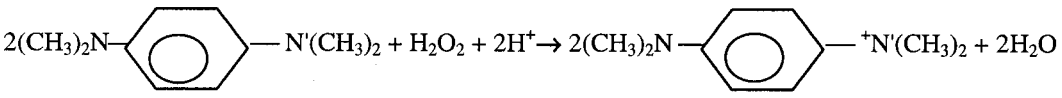
The potential for interaction of Cu,Zn-SOD with some other enzyme in amyotrophic lateral sclerosis can not be ruled out

In our screening for such a co-pathogenic enzyme, the preliminary data supported a synergistic cooperation between the peroxidatic activities of Cu,Zn-SOD and cytochrome c. The results were not implausible, because of the know peroxidatic activity of cytochrome c and the capacity for dramatic amplification of this peroxidatic activity in its degradation to microperoxidase. It was only after exhaustive attempts to replicate this initial finding that I finally concluded that the initial results were spurious. The Zn-depleted enzyme did not show any synergism with cytochrome c either. Adding small molecules also did not produce synergism (Figure 7, and 8). These negative findings, however, cannot rule out the possibility that agent(s) other than cytochrome c may have a synergistic or permissive role in releasing the toxic effects of mutant Cu,Zn-SOD.

Section 2: Peroxidatic activity of cytochrome c

Cytochrome c enhances TMPD peroxidation by forming a transition complex

H_2O_2 can readily convert TMPD to its oxidized product, Wurster's Blue:



Cytochrome c has a prosthetic heme group as do peroxidases, such as horseradish peroxidase, and catalase, whose functions are to catalyze the reaction of H_2O_2. Thus, it is no surprise that cytochrome c on its own has the capacity to catalyze the peroxidation. This was an early finding by Keilin, one of the initial discoverers of cytochrome c, and it has been replicated many times.[209,359,360,361] Figure 9 shows our confirmation using TMPD that cytochrome c, accelerates peroxidation reaction almost 10-fold.

If cytochrome c acts as an enzyme in TMPD peroxidatic oxidation, this reaction involves one enzyme (cytochrome c) and two substrates (TMPD and H_2O_2). Like most enzyme-catalyzed reactions, TMPD peroxidation catalyzed by cytochrome c follows a conventional process:[362] (1) the substrates bind to the enzyme to form a substrate(s)-enzyme complex, (2) the enzyme-substrate(s) complex transforms into enzyme-product(s) complex, and (3) the enzyme-product(s) complex breaks down to the product(s) and free enzyme.

Since the kinetics of this reaction had not previously been characterized, we set ourselves to investigate the more obvious characteristics of the reaction. The transition complex of

two-substrate reactants with an enzyme can be either a ternary complex or a binary complex. That only cytochrome c and H_2O_2, but not TMPD, obey the classic Michaelis-Menten saturation kinetics (Figure 12, and 13) suggests that TMPD peroxidation catalyzed by cytochrome c proceeds through a binary complex of cytochrome c and H_2O_2.[212] The straight line of the double logarithmic plot of reaction velocity against TMPD concentration (Figure 11) also indicates that TMPD might not be involved in forming the transition complex. Instead, TMPD might participate in the reaction as a "collision complex".

If cytochrome c, H_2O_2, and TMPD form a ternary complex, the rate of dissociation of TMPD from the complex must be greater than the formation of the ternary complex. The rapid dissociation of TMPD from the complex could also give the non-linear profile of the Lineweaver-Burk plot and a linear profile of the logarithmic plot of reaction velocity against TMPD concentration (Figure 11).

The second order reaction with respect to TMPD implies that two molecules of TMPD are involved prior to the rate-determining step. This is consistent with a reaction between a one-electron donor and a two-electron acceptor. The saturation kinetics with respect to H_2O_2 and to cytochrome c implies the formation of a complex between these two substrates (Figures 12 and 13).

The non-linear Arrhenius plot suggests a change from one rate-determining step to another. At temperatures above a transition point, a different reaction evidently becomes

rate determining. The effects of temperature show that the rate-determining step changes to one of higher activation energy at higher temperatures (Figure 14). This is unexpected for two reasons. First, thinking superficially, a faster reaction reflects a decreased free energy of activation. Since the Arrhenius activation energy is higher, it is not the enthalpy of activation that speeds the reaction. The increased rate must be driven by a more favorable *entropy* of activation.

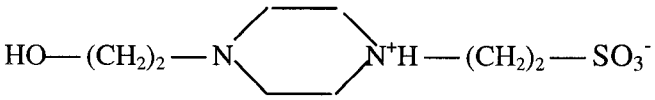
Influence of reaction conditions on peroxidatic activity of cytochrome c

Increasing temperature increases kinetic energy of reactants

Even though increasing the temperature sometimes increases the activation energy, as in the cytochrome c-catalyzed TMPD peroxidation, the overall effect of increasing temperature is an increase in reaction rate (Figure 14). The explanation of the effect of temperature is very simple. At higher temperatures, the probability that two molecules will collide is higher. Moreover, the higher collision velocity results in a higher kinetic energy, which allows more collisions to reach or exceed the activation energy of the reaction.

Effects of buffer may relate to the nature of the buffer

The effect of buffer on cytochrome c-catalyzed TMPD peroxidation depended on the buffering species, whether the buffer contains EDTA or not, and the concentration of the buffer (Figure 15). HEPES is a sterically hindered base and its structure is similar to that of TMPD.



Structure of HEPES

Consequently, HEPES might block or competitively bind cytochrome c and interfere with access of TMPD to cytochrome c. Alternatively, the presence of phosphate in potassium phosphate buffer may increase the reaction due to the known binding of phosphate by cytochrome c and its effect on the reduction potential.[363]

EDTA can facilitate redox cycling of transition metals

EDTA is widely used to sequester iron in biological model systems.[364,365,366,367] Nevertheless, the presence of EDTA accelerates the oxidation of ferrocytochrome c by hydrogen peroxide.[368,369] This observation is consistent with our date shown Figure 15. The increased oxidation of ferrocytochrome c would make more ferricytochrome c available to oxidize TMPD. In addition, since the EDTA-iron complex catalyzes the oxidation of ferrocytochrome c by H_2O_2, it might also catalyze the oxidation of TMPD by cytochrome c or H_2O_2. Furthermore, EDTA would bind any adventitious iron released from cytochrome c lowering its reduction potential[370] into the range appropriate for redox cycling, increasing the contribution of iron to the reaction. This, however, could not explain the observed activation during preincubation, since, as Figure 23 shows, equimolar or greater amounts of iron are not as active as the H_2O_2-modified cytochrome c.

Effect of pH on cytochrome c-catalyzed TMPD peroxidation

Every enzyme functions only over a limited range of pH and in most cases there is a definite (optimum) pH at which the enzyme has maximal catalytic capacity. This optimum may be due to a true reversible effect of pH on maximal velocity, on the binding affinity of substrate to the enzyme, or on the stability of the enzyme.[362]

An enzyme is made of many amino acids, among which some have ionizable side chains. Each exists in a state of ionization that depends on its dissociation constant and the pH of the solvent. Usually pH acts on the charge of ionizable components in the system. These include substrate, free enzyme, and enzyme-substrate complex. Changing the ionization of the free enzyme or substrate usually affects Km. On the other hand, changing the ionization of the enzyme-substrate or enzyme-product complex affects mainly the reaction velocity.[362]

Once substrate has bound at the catalytic site of enzyme, the ionized (or charged) functional groups of the side chains of nearby aminoacyl residues may modulate the catalysis through so-called acid or base catalysis.[371] Acid-base catalysis by enzyme can be either specific or general. If the rate of reaction changes as a function of pH at constant buffer concentration (as in the case of Figure 16), the reaction is specific base-catalyzed (if the pH is above 7) or specific acid-catalyzed (if the pH is below 7). If the rate of reaction changes as a function of buffer concentration at constant pH (as in the case of Figure 17), the reaction is general base-catalyzed (if the pH is above the pK of the buffer salt) or general acid-catalyzed (if the pH is below the pK of the buffer salt). By this

nomenclature, the specific acid catalyst is H^+ (strictly the hydronium ion H_3O^+) and the specific base catalyst is the hydrated HO^- ion.[371]

Increasing ionic strength stabilizes the ground state of reactants

The influence of salts on the reaction rate (Figure 17) can be explained in part as an effect of ionic strength. Increasing ionic strength stabilizes the ground state of reactants, by decreasing charge interactions. Consequently, the energy of the transition state is raised relative to the ground state, and the rate of reaction decreases.

Mechanism of actions of the halides are more complicated

The actions of the halides are not explicable on the basis of ionic strength alone. The purpose of addition of metal chelators is to clarify whether the halide was the effective agent, or whether the acceleration reflected the presence of trace metals in the buffer. Adding a metal chelator completely removed the enhancement by Na_2SO_4 that used as a non-halide control. Thus, the enhancement by Na_2SO_4 was due to the effect of trace metals in the reaction solution. F^- and Br^- follow the Na_2SO_4 pattern; in the presence of metal chelator, they had no effect on the reaction. Therefore, the enhancing effects of F^- and Br^- were due to trace metals in the reaction solution (Figure 18). Adding metal chelators to the reaction solution failed, however, to abolish the Cl- induced acceleration, instead enhancing the effect. This indicates that Cl^- itself can increase the peroxidatic activity of cytochrome c. As with phosphate, this may be due to the known binding of Cl^- by cytochrome c, which also has an effect on reduction potential.[363] The enhancement by I^- was not due to trace metals in the reaction solution because adding metal chelators failed to abolish the enhancing effect. It is not clear, however, why I^- had even stronger

effect than Cl^-, and what is the mechanism behind the difference that I^- increased both uncatalyzed and cytochrome c-catalyzed TMPD peroxidation (Figure 19) but Cl^- had not effect on uncatalyzed TMPD peroxidation (data not shown).

Effects of cations

The effects of Ca^{2+} require further investigation

Calcium is involved with proteins in blood clotting, enzyme regulation, transmitting chemical and electrical signals along nerves and muscles, releasing neurotransmitters, muscle contraction and blood pressure regulation. Calcium also plays an important role in intracellular regulation, and disturbances of calcium homeostasis can trigger cellular dysfunction, including the catastrophic collapse known as apoptosis,[372,373,374] potentially involved in diseases such as amyotrophic lateral sclerosis.[375,376]

Concentrations of calcium above the chelating capacity of the EDTA and other metal chelators might bind cytochrome c and change its conformation or stabilize against peroxidatic activation, resulting in a decrease in the reaction rate (Figure 20). The implication of this effect of calcium on the peroxidatic activity of cytochrome c is not clear. An increase in cytosolic calcium has been observed during apoptosis in thymocytes, and calcium ionophores reportedly induce apoptosis, [377,378,379] except in the presence of calcium inhibitors.[380] Increased cytosolic calcium reportedly activates calcium-dependent endonucleases and this activation is believed to be responsible for the DNA fragmentation that occurs during apoptosis.[381] The calcium-activated protease, calpain, also mediates apoptosis, stimulating production of reactive oxygen species. It is

tempting to speculate that during the apoptotic cascades, calpain might release peroxidatic activity by proteolytic scission of cytochrome c with the consequent production of microperoxidases.

Zn^{2+} inhibits TMPD peroxidation by blocking the generation of free radicals through Fenton reactions

Zinc, although a transition metal, behaves differently from iron and copper. Instead of enhancing the generation of free radicals via Fenton-type reactions, zinc reportedly acts as an antioxidant.[382,383] The simplest explanation of the zinc's inhibition of cytochrome c-catalyzed TMPD peroxidation (Figure 21) is that zinc is physically similar to Fenton reagent transition metals such as iron and copper, but redox-inactive. Adding zinc to the system can displace redox-active agents from their site-specific targets, decreasing the impact of Fenton-type reactions.

Cu^{2+}, Fe^{2+} and Fe^{3+} increase TMPD peroxidation by facilitating Fenton-type reactions

The lack of effect of $CuSO_4$ at 50 μM might be attributed to the chelating effect of EDTA in the buffer (Figure 22). It also indicates that levels of copper existing in the phosphate buffer do not interfere with the results if the buffer used contains EDTA as long as the concentration of copper is less than 50 μM.

At 75 μM $CuSO_4$ and above, the chelating capacity of 0.1 mM EDTA in the buffer might be exceeded. The extra copper could generate free radicals through Fenton-type reactions

and therefore facilitated both the uncatalyzed and cytochrome c-catalyzed TMPD peroxidation (Figure 22a).

Iron, added alone, had only slight peroxidatic activity, show the specific activity of H_2O_2-modified cytochrome c (Figure 23). The low peroxidatic activity of iron indicates that changes in the peroxidatic activity of cytochrome c induced by H_2O_2 cannot be attributed to release of free iron during incubation. The small amounts available, even if all the iron in cytochrome c were released would not be significant, in the system I studied.

Effects of free radical scavengers and antioxidants on cytochrome c-catalyzed TMPD peroxidation

Inhibition of peroxidatic activity of cytochrome c by benzoate, mannitol, and formate indicates the involvement of hydroxyl radicals in the reaction

The inhibition of hydroxyl radical scavengers benzoate, mannitol, and formate on uncatalyzed TMPD peroxidation, indicates the involvement of the hydroxyl radical in the reaction (Figure 24). The hydroxyl radical might also play a role in cytochrome c-catalyzed TMPD peroxidation because benzoate, mannitol and formate inhibited this reaction also. It is not clear, however, why benzoate inhibited the reaction more effectively at low concentration but less effectively at high concentration. Benzoate reportedly has only minimal redox interactions[384] while the scavenging of hydroxyl radical by formate results in generating CO_2^-, which, in the presence of O_2, produces $O_2^{\bullet-}$.[36]

Inhibition of peroxidatic activity of cytochrome c by urate may not be due to its hydroxyl radical scavenging effect

Urate is not only a hydroxyl radical scavenger but also a metal chelator. It also is an effective scavenger of oxo-heme species.[385] The low effect of urate might be due to the low concentration used relative to other hydroxyl radical scavengers (Figure 25a). Urate can only be dissolved in alkaline solution, so 750 μM was the highest concentration that could be used without changing the pH in the cuvet. Consequently, the inhibition by urate at concentration 1 mM of cytochrome c-catalyzed TMPD peroxidation when urate was added before pre-incubating cytochrome c with H_2O_2 may be in part by changing the pH of the pre-incubation mixture (Figure 25b). Alternatively, the inhibition by urate may be due to its metal chelating activity or scavenging an oxo-heme intermediate responsible for activation of the peroxidatic activity.

Superoxide radicals do not play a part in the reaction mechanism

Superoxide reportedly plays a role in TMPD autoxidation.[386] To find out whether superoxide plays a role in uncatalyzed or cytochrome c-catalyzed TMPD peroxidation I studied the effect of its scavenger, superoxide dismutase. There are two types of superoxide dismutase in humans: Cu,Zn-SOD and Mn-SOD.[387] Cu,Zn-SOD exists in the cytosol and extracellular fluids, while Mn-SOD exists in mitochondria.[387] H_2O_2 can inactivate Cu,Zn-SOD but has no effect on Mn-SOD.[388] Since H_2O_2 is a substrate of the reaction in our system, I used Mn-SOD to eliminate the confounding effect of H_2O_2 on enzyme activity. My study indicates that Mn-SOD had no effect on uncatalyzed or cytochrome c-catalyzed TMPD peroxidation over the tested concentration range of 12.5

to 50 U/ml (data not shown). This lack of effect indicates that superoxide is not significantly involved in the reactions prior to the rate-determining step.

Ascorbate inhibits peroxidatic activity for as long as ascorbate remains present in the reaction medium

Ascorbate, better known as vitamin C, is a powerful water-soluble antioxidant. It is an essential nutrient for humans. Biologically, the most important chemical property of ascorbate is its ability to act as an electron donor and react rapidly with free radicals.[389] In such reactions, ascorbate itself is oxidized to semidehydroascorbate radical, which can be disproportioned to dehydroascorbic acid and ascorbic acid.[390]

In cytochrome c-catalyzed TMPD peroxidation, the amount of free radicals generated from the reaction might overwhelm the scavenging capacity of ascorbate. The degree of overwhelming was directly related to the amount of ascorbate added to the system. The greater the amount of ascorbate added, the longer it took for all of the ascorbate to be oxidized to the semidehydroascorbate radical. This dose-response relationship explains the increased lag time of cytochrome c-catalyzed TMPD peroxidation when increasing amounts of ascorbate were added to the system. Alternatively, it could just be that the ascorbate merely stops the reaction until it is oxidized and then the reaction resumes.

The biphasic cytochrome c-catalyzed TMPD peroxidation observed when ascorbate was added, might relate to dual effect of ascorbate. Ascorbate not only acts as an antioxidant but also a pro-oxidant. [391] Ascorbate can reduce the transition metals and facilitate the Fenton reaction if H_2O_2 also exits in the system, leading to the generation of reactive

oxygen species.[392] Such an effect of ascorbate might explain its acceleration of TMPD peroxidation. Whether and when ascorbate acts an antioxidant or a pro-oxidant is dictated by many factors and a clear picture regarding this dichotomy is currently unavailable. Alternatively, the acceleration by ascorbate, as I will discuss later, might also relate to its effect on polymerization of cytochrome c (Figure 31).

Anthocyanin acts an antioxidant to inhibit peroxidatic activity of cytochrome c

Anthocyanins are natural colorants belonging to the flavonoid family responsible for the blue, purple, and red color of many plant tissues. The phenolic structure of anthocyanins conveys antioxidant activity in model systems via donation of electrons or hydrogen atoms from hydroxyl moieties to free radicals.[393] Like other plant polyphenols, many anthocyanins have marked antioxidant activity in vitro.[394,395]

The inhibition by crude extract of anthocyanins of cytochrome c-catalyzed TMPD peroxidation might result either from the antioxidant effect of anthocyanin itself, or from effects of impurities in the extract. In fact, the inhibition is attributable to the anthocyanin itself, because a purified anthocyanin, cyanidin-3-O-beta-glucopyranoside, had the same effect (Figure 27). The enhancement by crude extract of anthocyanin on uncatalyzed TMPD peroxidation at low concentration must come from other elements in the extract because pure anthocyanin had no such an effect.

Trolox inhibits cytochrome c-catalyzed TMPD peroxidation by scavenging a reaction intermediate

Trolox is commonly used as a reference or standard to compare the strength of antioxidants. Trolox blocks apoptosis induced by oxidative stress,[396] apoptosis in irradiated MOLT-4 lymphocytes,[397] peroxynitrite-mediated oxidative stress and apoptosis in rat thymocytes,[398] and protects against oxidative damage to kidney, heart, lung and spleen.[399] The inhibition by trolox of cytochrome c-catalyzed TMPD peroxidation in a dose-dependent manner confirms the antioxidant activities described in the abovementioned literature.

Trolox had no effect on the uncatalyzed TMPD peroxidation (Figure 28) suggests that the uncatalyzed and cytochrome c-catalyzed TMPD peroxidation might follow different pathways. In cytochrome c-catalyzed TMPD peroxidation, trolox presumably scavenges an intermediate that is missing in uncatalyzed TMPD peroxidation. A likely candidate for this intermediate is a cytochrome c radical generated in its reaction with H_2O_2.[400]

Figures 27 and 28 also show that anthocyanins are very powerful antioxidants. In our case, the anthocyanin's antioxidant capacity was at least 50 to 100-fold stronger than that of trolox when the same concentration was used.

Pre-incubation with H_2O_2 may change the conformation of cytochrome c and damage the heme chromophore

The effect of H_2O_2 incubation time on peroxidatic activity of cytochrome c is nearly unexplored territory in the field of cytochrome c research. My data show that pre-incubation has biphasic effects on cytochrome c-catalyzed TMPD peroxidation (Figure 29). The simplest explanation of this biphasic effect of incubation time on the reaction is the conformation change or structural damage to cytochrome c, caused by H_2O_2. The results of our SDS-PAGE electrophoresis (Figure 31) favor the former suggestion.

If the incubation time is less than 10 s, incubating with H_2O_2 may lead to opening or exposure of the binding or active site of cytochrome c for its substrates, or it may facilitate the breakdown of the enzyme-product(s) complex. In any case, the result is an increase in catalytic activity.

Breaking down the cytochrome c into fragments proteolytically yields microperoxidases that have stronger peroxidatic activity than the intact protein. As mentioned previously, the cytochrome c fragment 11-21 has 20 times as much peroxidase activity as native cytochrome c,[213] and the peroxidase activity of cytochrome c fragment 1-65 was 88-fold (pH 3.0) to 875-fold (pH 7.0) higher.[216] In the current studies, activation of peroxidatic activity by H_2O_2 involves partial bleaching of Sorêt absorbance, indicating a change to the heme chromophore (Figure 30).

Enhanced peroxidatic activity of H_2O_2 pre-incubated cytochrome c is not due to the microperoxidase of a cytochrome c fragment

The results of SDS-PAGE electrophoresis (Figure 31) suggest that the enhanced peroxidatic activity of cytochrome c after being incubated with H_2O_2 is not due to the microperoxidase of a cytochrome c fragment. Instead, the enhanced peroxidatic activity may arise from the polymerization or conformation change of the protein after being incubated with H_2O_2. As indicated previously, the enhancement by ascorbate of cytochrome c-catalyzed TMPD peroxidation at the second phase might relate to its enhancing activity on polymerization of cytochrome c. On the other hand, the inhibition by trolox and anthocyanin of cytochrome c-catalyzed TMPD peroxidation might be due to blocking the polymerization of cytochrome c. It would be appropriate to use Enzyme-Linked Immunosorbant Assay (ELISA) to quantify effects of ascorbate, trolox, and anthocyanins on polymerization of cytochrome c during incubation with H_2O_2. In doing so it can be confirmed at what stage these reagents are acting to inhibit peroxidation.

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Using TMPD oxidation as an index, this thesis explored the peroxidatic activities of superoxide dismutase and cytochrome c. Based on the current data I summarize my observations and conclusions as follows:

Cu,Zn-SOD has minimal peroxidatic activity

The native Cu,Zn-SOD did not express obvious peroxidatic activity in the current system and the release of its peroxidatic activity needs small molecules such as bicarbonate capable of entering the active site.

Removing zinc from Cu,Zn-SOD increases the peroxidatic activity of superoxide dismutase toward TMPD

The chemically modified Zn-depleted enzyme accelerated H_2O_2-induced oxidation of TMPD. Peroxidatic activity of the Zn-depleted enzyme is not dependent on addition of bicarbonate or other small molecules.

H_2O_2 activates the peroxidatic activity of cytochrome c

Pre-incubating cytochrome c with H_2O_2 increased the peroxidatic activity of cytochrome c with optimal incubation time around 10 s. Enhancement of the peroxidatic activity of cytochrome c by H_2O_2 was affected by experimental conditions such as temperature, ionic strength, pH, buffering species, and whether the buffer used contained the metal chelator, EDTA. Specifically, the peroxidatic activity of cytochrome c

increased with increasing temperature but decreased with increasing ionic strength, showed an optimal peroxidatic activity at pH 8.0, and was more active in phosphate buffer than in HEPES. Adding EDTA to the buffer increased the peroxidatic activity of cytochrome c by affecting the redox cycling of iron. The activation of the peroxidatic activity by H_2O_2 pre-incubation coincided with partial bleaching of the Sorêt band of cytochrome c, suggesting activation to a modified species.

Halides increase the peroxidatic activity of cytochrome c

The halides chloride and iodide (but not bromide and fluoride) increased the peroxidatic activity of cytochrome c. The enhancement by iodide was stronger compared with that by chloride. Iodide not only increased the peroxidatic activity of cytochrome c but also increased uncatalyzed TMPD peroxidation. In contrast, chloride had little such effect on uncatalyzed TMPD peroxidation, suggesting an effect on the activity of cytochrome c.

Calcium and zinc decrease the peroxidatic activity of cytochrome c

Both calcium and zinc decreased the peroxidatic activity of cytochrome c, but had little effect on the uncatalyzed TMPD peroxidation. Inhibition by calcium and zinc at μM levels suggest possible biological relevance.

Many antioxidants decrease the peroxidatic activity of cytochrome c

Antioxidants formate, mannitol, benzoate, anthocyanin, and trolox decreased the peroxidatic activity of cytochrome c. The effect of ascorbate on the peroxidatic activity of cytochrome c had two phases. Initially, ascorbate completely blocked the reaction, but after oxidation of ascorbate, the reaction accelerated to greater than control rates, showing generation of a reactive product.

Pre-incubation with H_2O_2 results in polymerization of cytochrome c

Pre-incubation of cytochrome c with H_2O_2 led to formation of a dimer, or even a trimer, of cytochrome c. Ascorbate seemed to enhance the polymerization effect of the H_2O_2 on cytochrome c while trolox and anthocyanin seemed to inhibit the polymerization. The results suggest that activation of the peroxidatic activity of cytochrome c coincides with inactivation of its respiratory electron-transfer activities.

Questions arising from this study and future directions

This study has answered some of the questions posed at the start, and inevitably raised others. One such question is whether impairment of the Zn-binding site allows for a positive feedback loop, and causes toxic activity significant in amyotrophic lateral sclerosis. Another question is whether the positive feedback in the activation of peroxidase activity of cytochrome c has significance in apoptosis. These new questions provide avenues for future study.

Since removing zinc from Cu,Zn-SOD increases its peroxidatic activity toward H_2O_2, and H_2O_2 treatment further damages the Zn-binding site of the enzyme[19], it is possible that this vicious cycle occurs in familial amyotrophic lateral sclerosis. In familial amyotrophic lateral sclerosis, the Zn-binding site of many mutant Cu,Zn-SODs is impaired to the point where affinity for zinc is decreased up to 30-fold relative to the wild-type.[17,20]

Therefore, there might be a positive feedback loop in amyotrophic lateral sclerosis because of the conformation change due to impairment of the Zn-binding site. A schematic process of such a positive feedback loop might look like this: mutation of Cu,Zn-SOD → impairment of Zn-binding site → diminished affinity of enzyme for zinc → conformational change of protein → increased access of abnormal substrate to the active site of enzyme → toxic non-dismutation activity → further damage to and impairment of the Zn-binding site. The issue of zinc affinity and enzyme instability is currently under investigation in other laboratories.

If the work of superoxide dismutase of this study is continued, I would like to study the peroxidatic activity of Cu,Zn-SOD at high concentration. The concentration in mammalian red blood cell is 500 U/ml and might be higher in the brain and motor neuron.[401] The negative data from the concentration of 50 U/ml in this study might not reflect the situation *in vivo*. I would also like to explore the peroxidatic activity of genetically modified Cu,Zn-SOD. For example, using site-directed mutagenesis, which substitutes specific amino acid residues genetically, we can create the mutant Cu,Zn-SOD genes identified in patients with familial amyotrophic lateral sclerosis. Then, we can amplify them with PCR, insert them into a vector, and transfer them to *E. Coli* or yeast

for expression. After extracting the gene protein products from *E. Coli*, or yeast, we can measure the peroxidase activities and other potential toxic effects of such generated enzymes.

For both Cu,Zn-SOD and cytochrome c, the human and non-human forms are remarkably similar, particularly in the metal binding and active site domains. The mutant human Cu,Zn-SOD expressed in transgenic mice produces neuromuscular atrophy[9] suggesting an overlap, not only in the functionality of the enzyme, but also in its pathological actions. Nevertheless, it is acknowledged that data from bovine Cu,Zn-SOD and horse heart cytochrome c might or might not reflect the properties of human forms of Cu,Zn-SOD and cytochrome c. Although such differences might cause us to miss effects that can only be seen in the human form, the data with zinc-depleted enzyme are consistent with the observations by other workers with human Cu,Zn-SOD. Further studies with human form of the enzyme should replicate most, if not all, of the current findings.

Since scission of cytochrome c leads to fragments that have increased peroxidase activity, we can use protease such as pepsin and trypsin to break Cu,Zn-SOD into fragments to see whether the resultant fragments have enhanced peroxidatic activity or other side effects.

Knowing the structures of enzyme often yields insight of its functions. X-ray crystallography approach can be applied to investigate the conformational change of Zn-depleted superoxide dismutase and other types of modified superoxide dismutase. Because the function of the enzyme is not merely to lower Ea for the catalyzed reaction,

but to prevent unwanted side reactions, it is of importance to study the change of profile of their capability to prevent unwanted side reactions. Kinetic studies of the modified Cu,Zn-SODs and data such as Vmax and Km from such studies might provide interesting information. It might be also interesting to transfer the Zn-depleted superoxide dismutase and fragments of Cu,Zn-SOD to animals to see whether it can cause amyotrophic lateral sclerosis in these animals.

The most interesting result of this study was that pre-incubation with H_2O_2 increased peroxidatic activity of cytochrome c and caused polymerization of cytochrome c. Our data show that the peroxidatic activity of cytochrome c reached its peak when cytochrome c was pre-incubated with H_2O_2 for only about 10 s. Did pre-incubating for only 10 s also cause the breakage of heme coordination and release of iron, and if so why did further incubation decrease the activity? If we could control the pre-incubation time more accurately, what result would be obtained? Would these results completely differ from the results that we observed when cytochrome c was pre-incubated with H_2O_2 for 10 s, or would it only change the amplitude of the results?

Alternatively, would decreasing the concentration of reagents and increasing the pre-incubation time yield the same results as at higher concentration of reagents with shorter pre-incubation time? The concentration of H_2O_2 *in vivo* is much lower than the concentration used in this study and the "pre-incubation" time is much longer than 10 s. Thus, the results of studies of lower concentration with longer pre-incubation time might reflect the situation *in vivo*. If a low steady-state concentration of H_2O_2 interacting with cytochrome c for a longer time increases the peroxidatic activity of cytochrome c, such

enhanced peroxidatic activity of cytochrome c might play a role in apoptosis. Detailed kinetic studies of activation by H_2O_2 under different reaction conditions might provide useful information for the potential role of peroxidatic activity of cytochrome c in apoptosis.

Treatment with H_2O_2 releases encapsulated cytochrome c from artificial mitochondrial membranes,[401] and results in the appearance of cytochrome c in the cytosol of Jurkat T lymphocytes within 2 hours.[402] It is of importance to study whether the release of cytochrome c from the membrane by H_2O_2 is related to activation of peroxidatic activity of cytochrome c by H_2O_2. If H_2O_2 in the cytosol can activate the peroxidatic activity of the relocated cytochrome c during apoptosis in a similar pattern as shown in this study, the increased peroxidatic activity of cytochrome c might serve as a trigger for a positive feedback loop. In addition, my results show that pre-incubation of cytochrome c with H_2O_2 resulted in polymerization of cytochrome c. Does the cytochrome c form a dimer or trimer *in vivo* in the presence of H_2O_2? If it does, what role might polymerization of cytochrome c play in apoptosis? Well-designed studies *in vivo* might provide answers for these questions.

Some cytochrome c fragments have stronger peroxidase activity compared with the intact enzyme. Does this phenomenon have anything to do with the fact that proteolytic cleavage of a limited number of cellular proteins is a central biochemical feature of apoptosis? Biochemical and genetic analyses of apoptosis have determined that intracellular proteases are key effectors of cell death pathways. In particular, early studies have pointed to the primacy of caspase proteases, as mediators of execution. Evidence is

accumulating that non-caspases (including cathepsins and calpains) also have roles in mediating and promoting cell death.[403,404] The question, in the light of the current data, is whether these proteases cause the fragmentation of cytochrome c and increase its toxic activity. Thus, among other proteases, calpain and cathepsins might be option for creating and testing the microperoxidase for cytochrome c and their roles in apoptosis.

We also can use site-directed mutagenesis to study the effect of the role of each specific amino acid residues in their structure and function. As in the study of Cu,Zn-SOD, the conformational changes of cytochrome c due to the genetically modification can be determined by X-ray crystallography. The study of functions of genetically modified cytochrome c might not only provide a profile of their capability to prevent unwanted side reactions but also uncover the potential toxic activity of the enzyme. The proteolytic products of cytochrome c and genetically modified cytochrome c by site-directed mutagenesis can be injected into the cytosol of cells to examine their apoptotic effects.

More general, future research on amyotrophic lateral sclerosis should focus on some of these unanswered questions. For example, is the delayed onset of amyotrophic lateral sclerosis due to the slow disappearance of resistance, or to the slow accumulation of a toxic threat? What is the trigger of the onset? Why is there a long latent period? Why are motor neurons the main target tissue of amyotrophic lateral sclerosis? Since the rapid progress after the onset indicates the involvement of a positive feedback, future studies also should search such a positive feedback. If the positive feedback indeed exists, what is it? Is the positive feedback the same in familial amyotrophic lateral sclerosis as in

sporadic amyotrophic lateral sclerosis? Is the positive feedback mediated by apoptosis, or prion, or disorder of the immune system, or something else? Why do both familial amyotrophic lateral sclerosis and sporadic amyotrophic lateral sclerosis produce the same symptoms and target the same neurons? If the mutation of Cu,Zn-SOD is the root of amyotrophic lateral sclerosis and disease is caused by the toxic gain-of-function, what is the nature of the gain-of-function? Are there any other genetic abnormalities in familial amyotrophic lateral sclerosis?

Regarding the research on apoptosis, it is important to investigate what causes the release of cytochrome c from mitochondria. In addition to the caspase pathway, does cytochrome c contribute to apoptosis by other activity (or site reaction)? Besides caspase, what are the roles of non-caspase proteases such as calpain and cathepsin in apoptosis? Do these enzymes increase the peroxidatic activity of cytochrome c, or other potential toxic activity, and contribute to its role in apoptosis. Does the relocation of cytochrome c cause a conformation change due to the different environment?

Studies searching for the positive feedback in both amyotrophic lateral sclerosis and apoptosis should focus on those irreversible steps of cascade. As in many biochemical pathways, these irreversible steps are not only the places for the futile cycle but also the spots for a positive feedback. The switch of these two pathways is controlled by flip-flop mechanisms. For example, if the two irreversible steps of cascade are activated at the same time, a futile cycle results. If only one of the irreversible steps is activated, the consequence is a positive feedback.

The current studies revealed further questions that well-designed experiments might answer and such experiments will in their turn raise more questions. This is how our knowledge grows. We might be able to put a period for this thesis, but the search for ultimate solutions to the problems addressed continues.

REFERENCES

1 Brunori, M., and Rotilio, G. (1984). Biochemistry of oxygen radical species. Methods in Enzymology 105:22-35.

2 Kim, S.M., and Kang, J.H. (1997). Peroxidative activity of human Cu,Zn-superoxide dismutase. Mol. Cells. 28:120-124.

3 McCord, J.M., and Fridovich, I. (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244:6049-6055.

4 Stryer, L. (1988). Biochemistry. New York: W.H. Freeman and Co., 3rd edition, pp 402-405.

5 Nantes, I.L., Faljioni-Alario, A., Vercesi, A.E., Santos, K.E., Bechara, E.J. (1998). Liposome effect on the cytochrome c-catalyzed peroxidation of carbonyl substrates to triplet species. Free Radical Biol Med. 25:546-553.

6 Radi, R., Turrens, J.F., and Freeman, B.A. (1991). Cytochrome c-catalyzed membrane lipid peroxidation by hydrogen peroxide. Arch. Biochem. Biophys. 288:118-125.

7 Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X., Rahmani, Z., Krizus, A., McKenna-Yasek, D., Cayabyab, A., Gaston, S.M., Bergh, R., Hung, W.Y., Bird, T., Deng, G., Mulder, D.W., Smyth, C., Lang, N.G., Soriana, E., Pericak-Vance, M.A., Haines, J., Rouleau, G.A., Fuesella, J.S., Horvitz, H.R., and Brown, R.H., Jr. (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362:59-62.

8 Andersen, P.M., Spitsyn, V.A., Makarov, S.V., Nilsson, L., Kravchuk, O.I., Bychkovskaya, L.S., and Marklund, S.L. (2001). The geographical and ethnic

distribution of the D90A Cu,Zn-SOD mutation in the Russian Federation. Amyotroph. Lateral Scler. Other Motor Neuron Disord. 2:63-69.

9 Gurney, M.E., Pu, H., Chui, A.Y., Dal Canto, M.C., Polchow, C.Y., Alexander, D.D., Caliendo, J., Hentati, A., Kwon, Y.W., Deng, H.X., Zhai, P., Sufit, R.L., and Siddique, T. (1994). Motor neuron degeneration in mice that express a human Cu/Zn superoxide dismutase mutation. Science 264:1772-1775.

10 Reaume, A.G., Elliot, J.L., Hoffman, E.K., Kowall, N.W., Ferrante, R.J., Siwek, D.F., Wilcox, H.M., Flood, D.G., Beal, M.F., Brown, R.H., Jr., Scott, R.W., and Snider, W.D. (1996). Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. Nat. Genet. 13:43-47.

11 Bowling, A.C., Barkowski, E.E., McKenna-Yasek, D., Sapp, P., Horvitz, H.R., Beal, M.F., and Brown, R.H., Jr. (1995). Superoxide dismutase concentration and activity of familial amyotrophic lateral sclerosis. J. Neurochem. 64:2366-2369.

12 Dal Canto, M.C., and Gurney, M.E. (1994). Development of central nervous system pathology in a murine transgenic model of human amyotrophic lateral sclerosis. Am. J. Pathol. 145:1271-1279.

13 Wiedau-Pazos, M., Goto, J.J., Rabizadeh, S., Gralla, E.B., Roe, J.A., Lee, M.K., Valentine, J.S., and Bredesen, D.E. (1996). Altered reactivity of superoxide dismutase in familial amyotrophic lateral sclerosis. Science 271:515-518.

14 Yim, M.B., Kang, J.H., Yim, H.S., Kwak, H.S., Chock, P.B., and Stadtman, E.R. (1996). A gain-of-function of an amyotrophic lateral sclerosis-associated Cu,Zn-superoxide dismutase mutant: An enhancement of free radical formation due to a decrease in Km for hydrogen peroxide. Proc. Natl. Acad. Sci. U. S. A. 93:5709-5714.

15 Yim, M.B., Kang, J.H., Yim, H.S., Kwak, H.S., Chock, P.B., and Stadtman, E.R. (1996). A gain-of-function of an amyotrophic lateral sclerosis-associated Cu,Zn-superoxide dismutase mutant: An enhancement of free radical formation due to a decrease in Km for hydrogen peroxide. Proc. Natl. Acad. Sci. U. S. A. 93:5709-5714.

16 Siddique, T., Deng, H.X. (1996). Genetic of amyotrophic lateral sclerosis, Hum. Mol. Genet.,- 5:1465-1051

17 Crow, J.P., Sampson, J.B., Zhuang, Y., Thompson, J.A., and Beckman, J.S. (1997). Decreased zinc affinity of amyotrophic lateral sclerosis-associated superoxide dismutase mutants leads to enhanced catalysis of tyrosine nitration by peroxynitrite. J. Neurochem. 69:1936-1944.

18 Tainer, J.A., Getzoff, E.D., Richardson, J.S., and Richardson, D.C. (1983). Structure and mechanism of copper, zinc superoxide dismutase. Nature 306:284-287.

19 Sampson, J.B., and Beckman, J.S. (2001). Hydrogen peroxide damages the Zn-binding site of zinc-deficient Cu,Zn superoxide dismutase. Arch. Biochem. Biophys. 392:8-13.

20 Lyons, T.J., Liu, H., Goto, J.J., Nersissian, A., Roe, J.A., Graden, J.A., Cafe, C., Ellerby, L.M., Bredesen, D.E., Gralla, E.B., and Valentine, J.S. (1996). Mutations in copper-zinc superoxide dismutase that cause amyotrophic lateral sclerosis alter the zinc-binding site and the redox behavior of the protein. Proc. Natl. Acad. Sci. U. S. A. 93:12240-12244.

21 Sankarapandi, S., and Zweier, J.L. (1999). Bicarbonate is required for the peroxidase function of Cu, Zn-superoxide dismutase at physiological pH. J. Biol. Chem. 274:1226-1232.

22 Beckman, J.S., Carson, M., Smith, C.D., and Koppenol, W.H. (1993). ALS, SOD and peroxynitrite. Nature 364:584.

23 Lui, X., Kim, N. C., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome c. Cell 96:147-157.

24 Zhivotovsky, B., Orrenium, S., Brustugun, O.T., and Doskeland, S.O. (1998). Injected cytochrome c induces apoptosis. Nature 391:449-450.

25 Brustugun, O.T., Fladmark, K.E., Doskeland, S.O., Orrenius, S., and Zhivotovsky, B. (1998). Apoptosis induced by microinjection of cytochrome c is caspase-dependent and is inhibited by Bcl-2. Cell Death Differ. 5:660-668.

26 Reed, J.C. (1997). Cytochrome c: Can't live with it - can't live without it. Cell 91:559-562.

27 Schendel, S., Montal, M., and Reed, J.C. (1998). Bcl-2 family proteins as ion-channel. Cell Death Differ. 5:372-380.

28 Antunes, F., and Cadenas, E. (2001). Cellular titration of apoptosis with steady state concentrations of H_2O_2: submicromolar levels of H_2O_2 induce apoptosis through Fenton chemistry independent of the cellular thiol state. Free Radic. Biol. Med. 30:1008-1018.

29 Stoian, I., Oros, A., and Moldoveanu, E. (1996). Apoptosis and free radicals. Biochem. Mol. Med. 59:93-97.

30 Das, N., Gupta, S., and Mazumdar, S. (2001). Direct observation of release of cytochrome c from lipid-encapsulated protein by peroxide and superoxide: a possible

mechanism for drug-induced apoptosis. Biochem. Biophys. Res. Commun. 286:311-314.

31 Mann, T., and Keilin, D. (1938). Haemocuprein and hepatocuprein, copper-protein compounds of blood and liver in mammals. Proc. Roy. Soc. (London) B126:303-315.

32 Mann, T., and Keilin, D. (1938). Haemocuprein, a copper-protein compound of red blood corpuscles. Nature Lond., 142:148.

33 Mohamed, M. S., and Greenberg, D. M. (1953). Isolation of purified copper protein from horse liver. J. Gen. Physiol. 37:433-439.

34 Kanematsu, S., and Asada, K. (1994). Superoxide dismutase. In: Molecular Aspects of Enzyme Catalysis, Fukui, T., and Soda, K., eds. Kodansha Ltd., Tokyo.

35 Asada, K., Kanematsu, S., Okada, S., and Hayakawa, T. (1980). Phylogenetic distribution of three types of superoxide dismutase in organisms and cell organelles. In: Chemical and Biochemical Aspects of Superoxide Dismutase. Bannister, J. V., and Hill, H.A. O., eds. Elsevier/North-Holland, New York.

36 Klug, D., Rabani, J., and Fridovich, I. (1972). A direct demonstration of the catalytic action of superoxide dismutase through the use of pulse radiolysis. J. Biol. Chem. 247:4839-4842.

37 Kanematsu, S., and Asada, K. (1978). Superoxide dismutase from an anaerobic photosynthetic bacterium, Chromatium vinosum. Arch. Biochem. Biophys. 185:473-482.

38 Lumsden, J., Cammack, R., and Hall, D.O. (1976). Purification and physicochemical properties of superoxide dismutase from two photosynthetic microorganisms. Biochim. Biophys. Acta 438:380-392.

39 Lavelle, F., McAdam, M.E., and Fielden, E.M. (1977). A pulse-radiolysis study of the catalytic mechanism of the iron-containing superoxide dismutase from Photobacterium leiognathi. Biochem. J. 161:3-11.

40 McAdam, M.E., Levelle, F., Fox, R.A., and Fielden, E.M. (1977). A pulse-radiolysis study of the manganese-containing superoxide dismutase from Bacillus stearothermophilus. Biochem. J. 165:81-87.

41 Youn, H.D., Kim, E.J., Roe, J.H., Hah, Y.C., and Kang, S.O. (1996). A novel nickel-containing superoxide dismutase from Streptomyces spp. Biochem. J. 318:889-896.

42 Fridovich, I. (1974). Superoxide dismutases. Adv. Enzymol. 41:33-97.

43 Carrico, R.J., and Deutsch, H.F. (1970). The presence of zinc in human cytocuprein and some properties of the apoprotein. J. Biol. Chem. 245:723-727.

44 Fielden, E.M., Roberts, P.B., Bray, R.C., Lowe, D.J., Mautner, G.N., Rotilio, G., and Calabrese, L. (1974). Mechanism of action of superoxide dismutase from pulse radiolysis and electron paramagnetic resonance. Evidence that only half the active sites function in catalysis. Biochem. J. 139:49-60.

45 Evans, H.J., Steinman, H.M., and Hill, R.L. (1974). Bovine erythrocyte superoxide dismutase. Isolation and characterization of tryptic, cyanogen bromide, and maleylated tryptic peptides. J. Biol. Chem. 249:7315-7325.

46 Steinman, H.M., Naik, V.R., Abernethy, J.L., and Hill, R.L. (1974). Bovine erythrocyte superoxide dismutase. Complete amino acid sequence. J. Biol. Chem. 249:7326-7338.

47 Abernethy, J.L., Steinman, H.M., and Hill, R.L. (1974). Bovine erythrocyte superoxide dismutase. Subunit structure and sequence location of the intrasubunit disulfide bond. J. Biol. Chem. 249:7339-7347.

48 Steinman, H.M. (1982). Superoxide dismutase: Protein chemistry and structure-function relationship. In: Superoxide Dismutase. Volume I. Oberley, L.W, ed. CRC Press, Inc. Boca Raton, Florida.

49 Barra, D., Martini, F., Bannister, J.V., Schinina, M.E., Rotilio, G., Bannister, W.H., and Bossa, F. (1980). The complete amino acid sequence of human Cu/Zn superoxide dismutase. FEBS Lett. 120:53-56.

50 Bannister, J.V., Bannister, W.H., and Rotilio, G. (1987). Aspects of the structure, function, and application of superoxide dismutase. CRC Critical Rev. Biochem. 22:111-180.

51 Steinman, H.M. (1978). The amino acid sequence of mangano superoxide dismutase from Escherichia coli. J. Biol. Chem. 253:8708-8720.

52 Brock, C.J., and Walker, J.E. (1980). Superoxide dismutase from Bacillus stearothermophilus. Complete amino acid sequence of a manganese enzyme. Biochemistry 19:2873-2882.

53 Harris, J.I., Auffret, A.D., Northrop, F.D., and Walker, J.E. (1980). Structural comparisons of superoxide dismutases. Eur. J. Biochem. 106:297-303.

54 Walker, J. E., Auffret, A. D., Brock, C.J., and Steinman, H.M. (1980). Structural comparisons of superoxide dismutases. In: Chemical and Biochemical Aspects of Superoxide Dismutase. Bannister, J. V., and Hill, H.A. O., eds. Elsevier/North-Holland, New York.

55 Richardson, DC. (1977). The three-dimensional structure of Cu,Zn superoxide dismutase. In: Superoxide and Superoxide Dismutase. Michelson, A. M., McCord, J.M., and Fridovich, I., eds. Academic Press, London.

56 Malinowski, D.P., and Fridovich, I. (1979). Subunit association and side-chain reactivities of bovine erythrocyte superoxide dismutase in denaturing solvents. Biochemistry 18:5055-5060.

57 Lepock, J.R., Frey, H.E., and Hallewell, R.A. (1990). Contribution of conformational stability and reversibility of unfolding to the increased thermostability of human and bovine superoxide dismutase mutated at free cysteines. J. Biol. Chem. 265:21612-21618.

58 Hough, M.A., and Hasnain, S.S. (1999). Crystallographic structures of bovine copper-zinc superoxide dismutase reveal asymmetry in two subunits: functionally important three and five coordinate copper sites captured in the same crystal. J. Mol. Biol. 287:579-592.

59 Roberts, V.A., Fisher, C.L., Redford, S.M., McRee, D.E., Parge, H.E., Getzoff, E.D., and Tainer, J.A. (1991). Mechanism and atomic structure of superoxide dismutase. Free Radic. Res. Commun. 12-13:269-278.

60 Zelko, I.N., Mariani, T.J., and Folz, R.J. (2002). Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic. Biol. Med. 33:337-349.

61 Tainer, J.A., Getzoff, E.D., Beem, K.M., Richardson, J.S., and Richardson, D.C. (1982). Determination and analysis of the 2 A-structure of copper, zinc superoxide dismutase. J. Mol. Biol. 160:181-217.

62 Parge, H. E., Getzoff, E.D., Scandella, C.S., Hallewell, R. A., and Tainer, J. A. (1986). Crystallographic characterization of recombinant human CuZn superoxide dismutase J. Biol. Chem. 261:16215-16218.

63 McAdam, M.E., Feilden, E.M., Lavelle, F., Calabrese, L., Cocco, D., and Rotilio, G. (1997). The involvement of the bridging imidazolate in the catalytic mechanism of action of bovine superoxide dismutase. Biochem. J. 167:271-274.

64 Merli, A., Rossi, G., Djinovic-Carugo, K., Bolognesi, M., Desideri, A., and Rotilio, G. (1995). Evidence for breaking of the active site dimetal cluster in Cu,Co superoxide dismutase upon copper reduction: a polarized absorption spectra study. Biochem. Biophys. Res. Commun. 210:1040-1044.

65 Blackburn, N.J., Hasnain, S.S., Binsted, N., Diakun, G.P., Garner, C.D., and Knowles, P.F. (1984). An extended-X-ray-absorption-fine-structure study of bovine erythrocyte superoxide dismutase in aqueous solution. Direct evidence for three-co-ordinate Cu(I) in reduced enzyme. Biochem. J. 219:985-990.

66 Ascone, I., Castaner, R., Tarricone, C., Bolognesi, M., Stroppolo, M.E., and Desideri, A. (1997). Evidence of his61 imidazolate bridge rupture in reduced crystalline Cu,Zn superoxide dismutase. Biochem. Biophys. Res. Commun. 241:119-121.

67 Hodgson, E.K., and Fridovich, I. (1975). The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: inactivation of the enzyme. Biochemistry 14:5294-5299.

68 Getzoff, E.D., Tainer, J.A., Weiner, P.K., Kollman, P.A., Richardson, J.S., and Richardson, D.C. (1983). Electrostatic recognition between superoxide and copper, zinc superoxide dismutase. Nature 306:287-290.

69 Sharp, K., Fine, R., and Honig, B. (1987). Computer simulations of the diffusion of substrate to an active site of an enzyme. Science 236:1460-1463.

70 Beyer, W.F., Jr., Fridovich, I., Mullenbach, G.T., and Halliwell, B. (1987). Examination of the role of arginine-143 in the human copper and zinc superoxide dismutase by site-specific mutagenesis. J. Biol. Chem. 262:11182-11187.

71 Bertini, I., Banci, L., Luchinat, C., and Hallewell, R.A. (1988). The exploration of the active-site cavity of copper-zinc superoxide dismutase. Ann. N. Y. Acad. Sci. 542:37-52.

72 Orr, W.C., and Sohal, R.S. (1994). Extension of life-span by overexpression of superoxide dismutase and catalase in Drosophila melanogaster. Science 263:1128-1130.

73 Omar, B.A., Gad, N.M., Jordan, M.C., Striplin, S.P., Russell, W.J., Downey, J.M., and McCord, J.M. (1990). Cardioprotection by Cu,Zn-superoxide dismutase is lost at high doses in the reoxygenated heart. Free Radic. Biol. Med. 9:465-471.

74 Nelson, S.K., Bose, S.K., and McCord, J.M. (1994). The toxicity of high-dose superoxide dismutase suggests that superoxide can both initiate and terminate lipid peroxidation in the reperfused heart. Free Radic. Biol. Med. 16:195-200.

75 Kono, Y., and Fridovich, I. (1982). Superoxide radical inhibits catalase. J. Biol. Chem. 257:5751-5754.

76 Blum, J., and Fridovich, I. (1985). Inactivation of glutathione peroxidase by superoxide radical. Arch. Biochem. Biophys. 240:500-508.

77 Monteiro, H.P., and Winterbourn, C.C. (1989). 6-Hydroxydopamine releases iron from ferritin and promotes ferritin-dependent lipid peroxidation. Biochem. Pharmacol. 38:4177-4182.

78 Lipton, S.A., Choi, Y.B., Pan, Z.H., Lei, S.Z., Chen, H.S., Sucher, N.J., Loscalzo, J., Singel, D.J., and Stamler, J.S. (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. Nature 364:626-632.

79 Chang, E.C., and Kosman, D.J. (1990). O_2-dependent methionine auxotrophy in Cu,Zn superoxide dismutase-deficient mutants of Saccharomyces cerevisiae. J. Bacteriol. 172:1840-1845.

80 Reveillaud, I., Kongpachith, A., Park, R., and Fleming, J.E. (1992). Stress resistance of Drosophila transgenic for bovine Cu,Zn superoxide dismutase. Free Radic. Res. Commun. 17:73-85

81 Michiels, C., Raes, M., Toussaint, O., and Remacle, J. (1994). Importance of Se-glutathione peroxidase, catalase, and Cu/Zn-SOD for cell survival against oxidative stress. Free Radic. Biol. Med. 17:235-248.

82 Gardner, R., Salvador, A., and Moradas-Ferreira, P. (2002). Why does SOD overexpression sometimes enhance, sometimes decrease, hydrogen peroxide production? a minimalist explanation. Free Radic. Biol. Med. 32:1351-1357.

83 Elroy-Stein, O., and Groner, Y. (1988). Impaired neurotransmitter uptake in PC12 cells overexpressing human Cu/Zn-superoxide dismutase--implication for gene dosage effects in Down syndrome. Cell 52:259-267

84 Avraham, K.B., Schickler, M., Sapoznikov, D., Yarom, R., and Groner, Y. (1988). Down's syndrome: abnormal neuromuscular junction in tongue of transgenic mice with elevated levels of human Cu/Zn-superoxide dismutase. Cell 54:823-829

85 Fridovich, I. (1975). Superoxide dismutases. Ann. Rev. Biochem. 44:147-159.

86 Klug-Roth, D., Fridovich, I., and Rabani, J. (1973). Pulse radiolytic investigations of superoxide catalyzed disproportionation. Mechanism for bovine superoxide dismutase. J. Am. Chem. Soc. 95:2786-2790.

87 Rotilio, G., Bray, R.C., and Fielden, E.M. (1972). A pulse radiolysis study of superoxide dismutase. Biochim. Biophys. Acta 268:605-609.

88 Rotilio, G., Rigo, A., and Calabrese, L. (1978). Recent developments on the active site structure and mechanism of bovine copper-and zinc-superoxide dismutase. In: Frontiers in Physiocochemical Biology. Pullman, B., ed. Academic Press, New York.

89 Fee, J.A. (1977). Structure-function relationships in superoxide dismutase. In: Superoxide and Superoxide Dismutase. Michelson, A. M., McCord, J.M., and Fridovich, I., eds. Academic Press, London.

90 Rigo, A., Viglino, P., and Rotilio, G. (1975). Kinetic study of O_2 dismutation by bovine superoxide dismutase. Evidence for saturation of the catalytic sites by O_2. Biochem. Biophys. Res. Commun. 63:1013-1018.

91 Fee, J.A., and Gaber, B.P. (1972). Anion binding to bovine erythrocyte superoxide dismutase. Evidence for multiple binding sites with qualitatively different properties. J. Biol. Chem. 247:60-65.

92 Gaber, B.P., Brown, R.D., Koenig, S.H., and Fee, J.A. (1972). Nuclear magnetic relaxation dispersion in protein solutions. V. Bovine erythrocyte superoxide dismutase. Biochim. Biophys. Acta 271:1-5.

93 Fee, J.A., and Ward, R.L. (1976). Evidence for a coordination position available to solute molecules on one of the metals at the active center of reduced bovine superoxide dismutase. Biochem. Biophys. Res. Commun. 71:427-437.

94 Osman, R. (1986). Effect of local environment and protein on the mechanism of action of superoxide dismutase. Enzyme 36:32-43.

95 Rotilio, G., Calabrese, L., Mondovi, B., and Blumberg, W.E. (1974). Electron paramagnetic resonance studies of cobalt-copper bovine superoxide dismutase. J. Biol. Chem. 249:3157-3160.

96 Lawrence, G.D., and Sawyer, D.T. (1979). Potentiometric titrations and oxidation-reduction potentials of manganese and copper-zinc superoxide dismutases. Biochemistry 18:3045-3050.

97 Morpurgo, L., Giovagnoli, C., and Rotilio, G. (1973). Studies of the metal sites of copper proteins. V. A model compound for the copper site of superoxide dismutase. Biochim. Biophys. Acta 322:204-210.

98 Halliwell, B., and Gutteridge, J. (1989). Free Radicals in Biology and Medicine. Oxford: Clarendon Press. P86-106.

99 Fridovich, I. (1986). Superoxide dismutases. Adv. Enzymol. Relat. Areas Mol. Biol. 58:61-97.

100 Jones, D.P., Eklow, L., Thor, H., and Orrenius, S. (1981). Metabolism of hydrogen peroxide in isolated hepatocytes: relative contributions of catalase and glutathione peroxidase in decomposition of endogenously generated H_2O_2. Arch. Biochem. Biophys. 210:505-516.

101 Verkerk, A., and Jongkind, J.F. (1992). Vascular cells under peroxide induced oxidative stress: a balance study on in vitro peroxide handling by vascular endothelial and smooth muscle cells. Free Radic. Res. Commun. 17:121-132.

102 Scarlato, G. (1997). Amyotrophic lateral sclerosis: from pathological mechanisms to patient care. J. Neurol. 244(Suppl 2): S1-S2.

103 Julien, J.P. (2001). Amyotrophic lateral sclerosis. Unfolding the toxicity of the misfolded. Cell 104:581-591.

104 Charcot J.M. (1874). Lecons sur les maladies du systeme nerveux failtes a la Salpetriere. Progr. Med. (Paris) pp 213-242.

105 Cudkowicz, M.E., McKenna-Yasek, D., Sapp, P.E., Chin, W., Geller, B., Hayden, D.L., Schoenfeld, D.A., Hosler, B.A., Horvitz, H.R., and Brown, R.H. (1997). Epidemiology of mutations in superoxide dismutase in amyotrophic lateral sclerosis. Ann. Neurol. 41:210-221.

106 Andersen, P.M., Nilsson, P., Keranen, M.L., Forsgren, L., Hagglund, J., Karlsborg, M., Ronnevi, L.O., Gredal, O., and Marklund, S.L. (1997). Phenotypic heterogeneity in motor neuron disease patients with CuZn-superoxide dismutase mutations in Scandinavia. Brain 120:1723-1737.

107 Andersen, P.M., Nilsson, P., Ala-Hurula, V., Keranen, M.L., Tarvainen, I., Haltia, T., Nilsson, L., Binzer, M., Forsgren, L., and Marklund, S.L. (1995). Amyotrophic lateral sclerosis associated with homozygosity for an Asp90Ala mutation in CuZn-superoxide dismutase. Nat. Genet. 10:61-66.

108 Al-Chalabi, A., Andersen, P.M., Chioza, B., Shaw, C., Sham, P.C., Robberecht. W., Matthijs, G., Camu, W., Marklund, S.L., Forsgren, L., Rouleau, G., Laing, N.G., Hurse, P.V., Siddique, T., Leigh, P.N., and Powell, J.F. (1998). Recessive amyotrophic lateral sclerosis families with the D90A Cu,Zn-SOD mutation share a common founder: evidence for a linked protective factor. Hum. Mol. Genet. 7:2045-2050.

109 Robberecht, W., Aguirre, T., Van den Bosch, L., Tilkin, P., Cassiman, J.J., and Matthijs, G. (1996). D90A heterozygosity in the Cu,Zn-SOD gene is associated with familial and apparently sporadic amyotrophic lateral sclerosis. Neurology 47:1336-1339.

110 Deng, H.X., Hentati, A., Tainer, J., Iqbal, Z., Cayabyab, A., Hung, W.Y., Getzoff, E. D., Hu, P., Herzfeldt, B., Roos, R. P., Warner, C., Deng, G., Soriano, E., Smyth, C., Parge, H. E., Ahmed, A., Roses, A. D., Hallewell, R. A., Pericak-Vance, M. A., and Siddique, T. (1993). Amyotrophic lateral sclerosis and structural defects in Cu,Zn superoxide dismutase. Science 261:1047-1051.

111 Rosen, D.R., Bowling, A.C., Patterson, D., Usdin, T.B., Sapp, P., Mezey, E., McKenna-Yasek, D., O'Regan, J., Rahmani, Z., and Ferrante, R.J. (1994). A frequent ala 4 to val superoxide dismutase-1 mutation is associated with a rapidly progressive familial amyotrophic lateral sclerosis. Hum. Mol. Genet. 3:981-987.

112 Juneja, T., Pericak-Vance, M.A., Laing, N.G., Dave, S., and Siddique, T. (1997). Prognosis in familial amyotrophic lateral sclerosis: progression and survival in patients with glu100gly and ala4val mutations in Cu,Zn superoxide dismutase. Neurology 48:55-57.

113 McConkey, E.H. (1993). Human Genetics: The Molecular Revolution. Jones and Bartlett Publishers, Boston. USA.

114 Maroni, G. (2001). Molecular and Genetic Analysis of Human Traits. Blackwell Science, Inc., Massachusetts, USA.

115 Rabizadeh, S., Gralla, E.B., Borchelt, D.R., Gwinn, R., Valentine, J.S., Sisodia, S., Wong, P., Lee, M., Hahn, H., and Bredesen, D.E. (1995). Mutations associated with amyotrophic lateral sclerosis convert superoxide dismutase from an antiapoptotic gene to a proapoptotic gene: Studies in yeast and neural cells. Proc. Natl. Acad. Sci. U. S. A. 92:3024-3028.

116 Goto, J.J., Gralla, E.B., Valentine, J.S., and Cabelli, D.E. (1998). Reactions of hydrogen peroxide with familial amyotrophic lateral sclerosis mutant human copper-zinc superoxide dismutases studied by pulse radiolysis. J. Biol. Chem. 273:30104-30109.

117 Liochev, S.I., Chen, L.L., Hallewell, R.A., and Fridovich I. (1997). Superoxide-dependent peroxidase activity of H48Q: a superoxide dismutase variant associated with familial amyotrophic lateral sclerosis. Arch. Biochem. Biophys. 346:263-268

118 Bogdanov, M.B., Ramos, L.E., Xu, Z., and Beal, M.F. (1998). Elevated "hydroxyl radical" generation in vivo in an animal model of amyotrophic lateral sclerosis. J. Neurochem. 71:1321-1324.

119 Ferrante, R.J., Browne, S.E., Shinobu, L.A., Bowling, A.C., Baik, M.J., MacGarvey, U., Kowall, N.W., Brown, R.H., Jr, and Beal, M.F. (1997). Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis.J. Neurochem. 69:2064-2074.

120 Jewett, S.L., Rocklin, A.M., Ghanevati, M., Abel. J.M., and Marach, J.A. (1999). A new look at a time-worn system: oxidation of CuZn-SOD by H_2O_2. Free Radic. Biol. Med. 26:905-818.

121 Uchida, K., and Kawakishi, S. (1994). Identification of oxidized histidine generated at the active site of Cu,Zn-superoxide dismutase exposed to H_2O_2. Selective generation of 2-oxo-histidine at the histidine 118. J. Biol. Chem. 269:2405-2410.

122 Liochev, S.I., Chen, L.L., Hallewell, R.A., and Fridovich, I. (1998). The familial amyotrophic lateral sclerosis-associated amino acid substitutions E100G, G93A, and G93R do not influence the rate of inactivation of copper- and zinc-containing superoxide dismutase by H_2O_2. Arch. Biochem. Biophys. 352:237-239.

123 Estevez, A.G., Crow, J.P., Sampson, J.B., Reiter, C., Zhuang, Y., Richardson, G.J., Tarpey, M.M., Barbeito, L., and Beckman, J.S. (1999). Induction of nitric oxide-dependent apoptosis in motor neurons by zinc-deficient superoxide dismutase. Science 286:2498-2500.

124 Shibata, N., Hirano, A., Yamamoto, T., Kato, Y., and Kobayashi, M. (2000). Superoxide dismutase-1 mutation-related neurotoxicity in familial amyotrophic lateral sclerosis. Amyotroph. Lateral Scler. Other Motor Neuron Disord.1:143-161.

125 Torreilles, F., Salman-Tabcheh, S., Guerin, M., Torreilles, J. (1999). Neurodegenerative disorders: the role of peroxynitrite. Brain Res. Rev. 30:153-163.

126 Beal, M.F., Ferrante, R.J., Browne, S.E., Matthews, R.T., Kowall, N.W., and Brown, R.H., Jr. (1997). Increased 3-nitrotyrosine in both sporadic and familial amyotrophic lateral sclerosis. Ann. Neurol. 42:644-654.

127 Bruijn, L.I., Beal, M.F., Becher, M.W., Schulz, J.B., Wong, P.C., Price, D.L., and Cleveland, D.W. (1997). Elevated free nitrotyrosine levels, but not protein-bound nitrotyrosine or hydroxyl radicals, throughout amyotrophic lateral sclerosis-like disease implicate tyrosine nitration as an aberrant in vivo property of one familial ALS-linked superoxide dismutase 1 mutant. Proc. Natl. Acad. Sci. U. S. A. 94:7606-7611.

128 Abe, K., Pan, L.H., Watanabe, M., Kato, T., and Itoyama, Y. (1995). Induction of nitrotyrosine-like immunoreactivity in the lower motor neuron of amyotrophic lateral sclerosis. Neurosci. Lett. 199:152-154.

129 Strong, M.J., Sopper, M.M., Crow, J.P., Strong, W.L., and Beckman, J.S. (1998). Nitration of the low molecular weight neurofilament is equivalent in sporadic amyotrophic lateral sclerosis and control cervical spinal cord. Biochem. Biophys. Res. Commun. 248:157-164.

130 Williamson, T.L., Corson, L.B., Huang, L., Burlingame, A., Liu, J., Bruijn, L.I., and Cleveland, D.W. (2000). Toxicity of ALS-linked Cu,Zn-SOD mutants. Science 288:399.

131 Facchinetti, F., Sasaki, M., Cutting, F.B., Zhai, P., MacDonald, J.E., Reif, D., Beal, M.F., Huang, P.L., Dawson, T.M., Gurney, M.E., and Dawson, V.L. (1999). Lack of involvement of neuronal nitric oxide synthase in the pathogenesis of a transgenic mouse model of familial amyotrophic lateral sclerosis. Neuroscience 90:1483-1492.

132 Hodgson, E.K., and Fridovich, I. (1975). The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: chemiluminescence and peroxidation. Biochemistry 14:5299-5303.

133 Cabelli, D.E., Allen, D., Bielski, B.H, and Holcman, J. (1989). The interaction between Cu(I) superoxide dismutase and hydrogen peroxide. J. Biol. Chem. 264:9967-9971.

134 Bray, R.C., Cockle, S.H., Fielden, E.M., Roberts, P.B., Rotilio, G., and Calabrese, L. (1974). Reduction and inactivation of superoxide dismutase by hydrogen peroxide. Biochem. J. 139:43-48.

135 Blech, D.M., and Borders, C.L., Jr. (1983). Hydroperoxide anion, HO-2, is an affinity reagent for the inactivation of yeast Cu,Zn superoxide dismutase: modification of one histidine per subunit. Arch. Biochem. Biophys. 224:579-586.

136 Borders, C. L., Jr., and Fridovich, I. (1985). A comparison of the effects of cyanide, hydrogen peroxide, and phenylglyoxal on eucaryotic and procaryotic Cu,Zn superoxide dismutases. Arch. Biochem. Biophys. 241:472-476.

137 Salo, D.C., Pacifici, R.E., Lin, S.W., Giulivi, C., and Davies, K.J.A. (1990). Superoxide dismutase undergoes proteolysis and fragmentation following oxidative modification and inactivation. J. Biol. Chem. 265:11919-11927.

138 Yim, M.B., Chock, P.B., and Stadtman, E.R. (1990). Copper, zinc superoxide dismutase catalyzes hydroxyl radical production from hydrogen peroxide. Proc. Natl. Acad. Sci. U. S. A. 87:5006-5010.

139 Yim, M.B., Chock, P.B., and Stadtman, E.R. (1993). Enzyme function of copper, zinc superoxide dismutase as a free radical generator. J. Biol. Chem. 268:4099-4105.

140 Sankarapandi, S., and Zweier, J.L. (1999). Evidence against the generation of free hydroxyl radicals from the interaction of copper,zinc-superoxide dismutase and hydrogen peroxide. J. Biol. Chem. 274:34576-34583.

141 Konningsberger, J.C., van Asback, B.S., van Faassen, E., Wiegman, L.J.J.M., van Hattum, J., van Berge Henegouwen, G. P., and Marx, J. J. M. (1994). Copper, zinc-superoxide dismutase and hydrogen peroxide: a hydroxyl radical generating system. Clin. Chim. Acta 230:51-61.

142 Scott, R.A., and Mauk, A.G. (1996). Cytochrome c: A Multidisciplinary Approach. University Science Books, Sausalito, California.

143 Ambler, R.P. (1991). Sequence variability in bacterial cytochromes c. Biochim. Biophys. Acta 1058:42-47.

144 Dumont, M.E., Ernst, J.F., Hampsey, D.M. and Sherman, F. (1987). Identification and sequence of the gene encoding cytochrome c heme lyase in the yeast Saccharomyces cerevisiae. EMBO J. 6:235-241.

145 Nicholson, D.W., Kohler, H., and Neupert, W. (1987). Import of cytochrome c into mitochondria: cytochrome c heme lyase. Eur. J. Biochem. 164:147-157.

146 Drygas, M.E., Lambowitz, A.M., and Nargang, F.E. (1989). Cloning and analysis of the Neurospora crassa gene for cytochrome c heme lyase. J. Biol. Chem. 264:17897-17906.

147 Stuart, R.A., and Neupert, W. (1990). Apocytochrome c: an exceptional mitochondrial precursor protein using an exceptional import pathway. Biochimie. 72:115-121.

148 Moore, G. R., and Pettigrew, G.W. (1990). Cytochrome c. Evolutionary, Structural, and Physicochemical Aspects. New York: Springer-Verlag.

149 Wu, T.T., and Kabat, E.A. (1970). An analysis of the sequences of the variable region of Bence-Jones proteins and myoloma light chain and their implications for antibody complementarity. J. Exp. Med. 312:211-250.

150 Mathews, S. (1985). The structure, function and evolution of cytochromes. Prog. Biophys. Molec. Biol. 45:1-56.

151 Henning, B., and Neupert, W. (1981). Assembly of cytochrome c - apocytochrome c is bound to specific sites on mitochondria before it is converted to holocytochrome c. Eur. J. Biochem. 121:203-212.

152 Henning, B., Koehler, H., and Neupert, W. (1983). Receptor sites involved in posttranslational transport of apocytochrome c into mitochondria – specificity and number of sites. Proc. Natl. Acad. Sci. U. S. A. 80:4963-4967.

153 Louie, G.V., and Brayer, G.D. (1990). High-resolution refinement of yeast iso-1-cytochrome c and comparisons with other eukaryotic cytochrome c. J. Mol. Biol. 214:527-555.

154 Berghuis, A.M., and Brayer, G.D. (1992). Oxidation state-dependent conformation changes in cytochrome c. J. Mol. Biol. 223:959-976.

155 Murphy, M.E.P., Nall, B.T., and Brayer, G.D. (1992). Structure determination and analysis of yeast iso-2-cytochrome c and a composite mutant protein. J. Mol. Biol. 227:160-176.

156 Takano, T., and Dickerson, R.E. (1981). Conformation change of cytochrome c. I. Ferrocytochrome c structure refined at 1.5 Å resolution. J. Mol. Biol. 153:79-94.

157 Takano, T., and Dickerson, R.E. (1981). Conformation change of cytochrome c II. Ferricytochrome c structure refined at 1.8 Å and comparison with the ferrocytochrome c. J. Mol. Biol. 153:95-115.

158 Bushenell, G.W., Louie, G.V., and Brayer, G.D. (1990). High-resolution three-dimensional structure of horse heart cytochrome c. J. Mol. Biol. 214:585-595.

159 Ochi, H., Hata, Y., Tanaka, N., Kakudo, M., Sakuri, T., Achara, S., and Morita, Y. (1983). Structure of rice ferricytochrome c at 2.0 Å resolution. J. Mol. Biol. 166:407-418.

160 Dickerson, R.E., Kopka, M.L., Borders, C.L., Jr, Varnum, J., and Weinzier, J.E. (1967). A centrosymmetric projection at 4A of horse heart oxidized cytochrome c. J. Mol. Biol. 29:77-95.

161 Margoliash, E., and Schejter, A. (1966). Cytochrome c. Advan. Protein Chem. 21:113-286.

162 Sheriff, S., Hendrickson, W.A., and Smith, J.L. (1987). Structure of myo hemerythrin in the azidomet state at 1.7/1.3 Å resolution. J. Mol. Biol. 197:273-296.

163 McGregor, M.J., Islam, S.A., and Sternberg, M.J.E. (1987). Analysis of the relationship between side-chain conformation and secondary structure in globular protein. J. Mol. Biol. 198:295-310.

164 Twitchett, M.B. (1998). Electron Transfer Properties of Flavin-Modified and Axial Ligand Variants of Cytochrome c. The University of British Columbia, Vancouver, B.C., Canada. Ph.D Thesis.

165 Brayer, G.D., and Murphy, M.E.P. (1996). Structural studies of eukaryotic cytochrome c. In: Cytochrome c: A Multidisciplinary Approach. Scott, R. A., and Mauk, A.G., eds. University Science Books, Sausalito, pp. 103-166.

166 Dolphin, D. (1979). The Porphyrins. Academic Press, New York. Vol. VII, Part B, pp.241-289.

167 Louie, G.V., Hutcheon, W., and Brayer, G.G. (1988). Yeast iso-1-cytochrome c: a 2.8 Å resolution three-dimensional structure determination. J. Mol. Biol. 199: 295-314.

168 Qi, P.X., Urbauer, J.L., Fuentes, E.J., Leopold, M.F., and Wand, A.J. (1994). Structural water in oxidized and reduced horse heart cytochrome c. Nature Struct. Biol. 1:378.

169 Qi, P.X., Di Stefano, D.L., and Wand, A.J. (1994). Solution structure of horse heart ferrocytochrome c determined by high-resolution NMR and restrained simulated annealing. Biochemistry 33:6408-6417.

170 Qi, P.X., Beckman, R.A., and Wand, A.J. (1996). Solution structure of horse heart ferricytochrome c and detection of redox-related structural changes by high-resolution 1H NMR. Biochemistry 35:12275-12286.

171 Banci, L., Bertini, I., Gray, H.B., Luchinat, C., Reddig, T., Rosato, A., and Turano, P. (1997). Solution structure of oxidized horse heart cytochrome c. Biochemistry 36:9867-9877.

172 Banci, L., Bertini, I., Huber, J.G. Spyroulias, A., and Turano, P. (1999). Solution structure of reduced horse heart cytochrome c. JBIC 4:21-31.

173 Kar, L., Sherman, S.A., and Johnson, M.E. (1994). Comparison of protein structures in solution using local conformations derived from NMR data: application to cytochrome c. J. Biomol. Struct. Dyn. 12:527-558.

174 Calvert, J.F., Hill, J.L., and Dong, A. (1997). Redox-dependent conformational changes are common structural features of cytochrome c from various species. Arch. Biochem. Biophys. 346:287-293.

175 Fisher, W.R., Taniuchi, H., and Anfinsen, C.B. (1973). On the role of the heme in the formation of the structure of cytochrome c. J. Biol. Chem. 248:3188-3195.

176 Eden, D., Matthew, J. B., Rosa, J. J., and Richards, F.M. (1982). Increase in apparent compressibility of cytochrome c upon oxidation. Proc. Natl. Acad. Sci. U. S. A. 79:815-819.

177 Takano, T., and Dickerson, R.E. (1980). Redox conformation changes in refined tuna cytochrome c. Proc. Natl. Acad. Sci. U. S. A. 77:6371-6375.

178 David, L.A., Schejter, A., and Hess, G.P. (1974). Alkaline isomerization of oxidized cytochrome c. J. Biol. Chem. 249:2624-2632.

179 Rodkey, F.L., and Ball, E.G. (1950). Oxidation-reduction potentials of the cytochrome c system. J. Biol. Chem. 182:17-28.

180 Gadsby, P. M.A., Peterson, J., Foote, N., Greenwood, C., and Thomson, A.J. (1987). Identification of the ligand – exchange process in the alkaline transition of horse heart cytochrome c. J. Mol. Biol. 197:525-541.

181 Redfield, A.G., and Gupta, R.K. (1971). Pulsed NMR study of the structure of cytochrome c. Cold Spring Harbor Symp. Quant. Biol. 36:405-411.

182 Gupta, R.K., and Koenig, S.H. (1971). Some aspects of pH and temperature dependence of the NMR spectra of cytochrome c. Biochem. Biophys. Res. Commun. 45:1134-1143.

183 Dolphin, D. (1979). The Porphyrins. Academic Press, New York. Vol. III, Part A, pp.167-209.

184 Pettigrew, G. W., and Moore, G. R. (1987). Cytochrome c: Biological Aspects. Springer-Verlag, New York.

185 Margalit, R., and Schejter, A. (1973). Cytochrome c: a thermodynamic study of the relationships among oxidation state, ion binding and structural parameters I: The effects of temperature, pH and electrostatic media on the standard redox potential of cytochrome c. Eur. J. Biochem. 32:492-499.

186 Marchon, J.C., Mashiko, T., and Reed, C.A. (1982).How does nature control cytochrome redox potentials? In: Electron Transport and Oxygen Utilization. Ho, C., ed. Elsevier, Amsterdam, pp. 67-72.

187 Moore, G.R., and Williams, R. J. P. (1977). Structure basis for the variation in redox potential of c cytochromes. FEBS Lett. 79:229-232.

188 Moore, G.R., Pettigrew, G.W., and Rogers, N. K. (1986). Factors influencing redox potentials of electron transfer proteins. Proc. Natl. Acad. Sci. U. S. A. 83:4998-4999.

189 Cowgill, R.W., and Clark, W.M. (1952). Coordination of imidazoles with ferrimesoporphyrin. J. Biol. Chem. 198:33-61.

190 Falk, J. E. (1964). Porphyrins and Metalloporphyrins. Elsevier, Amsterdam.

191 Raphael, A.L., and Gray, H.B. (1989). Axial ligand replacement in horse heart cytochrome c by semisynthesis. Proteins 6:338-340.

192 Kassner, R.J. (1972). Effect of nonpolar environments on the redox potentials of heme complexes. Proc. Natl. Acad. Sci. U. S. A. 69:2263-2267.

193 Stellwagen, E. (1978). Heme exposure as the determinant of oxidation-reduction potential of heme protein. Nature Lond. 275:73-74.

194 Ito, A. (1980). Cytochrome b5 – like hemoprotein of outer mitochondrial membranes O.M. cytochrome b. I: Purification of O.M. cytochrome b from rat liver mitochondrial and comparison of its molecular properties with those of cytochrome b5. J. Biochem. 87:63-71.

195 Ito, A. (1980). Cytochrome b5 – like hemoprotein of outer mitochondrial membranes; O.M. cytochrome b II: Contribution of O.M. cytochrome b to rotenone in sensitive NADH-cyt c reductase activity. J. Biochem. 87:73-83.

196 Mcleod, R. M., Farkas, W., Fridovich, I., and Handler, P. (1961). Purification and properties of heparic sulfite oxidase. J. Biol. Chem. 238:1841-1846.

197 Bach, S.J., Dixon, M., and Keilin, D. (1942). A new soluble cytochrome component from yeast. Nature Lond. 149:21.

198 Bach, S.J., Dixon, M., and Zerfas, L.G. (1942). Lactic dehydrogenase of yeast. Nature Lond. 149:48-49.

199 Altschul, A.M., Abrams, R., and Hogness, T.R. (1940). Cytochrome c peroxidase. J. Biol. Chem. 136:777-794.

200 Margoliash, E., and Bosshard, H.R. (1983). Guided by electrostatics. A textbook protein comes of age. TIBS 8:316-320.

201 Morton, R.A., Overnell, J., and Harbury, H.A. (1970). Electron transfer between cytochrome c from horse and pseudomonas. J. Biol. Chem. 245:4653-4657.

202 Ewall, R.X., and Bennett, L.E. (1974). Reactivity characteristics of cytochrome c (III) adduced from its reduction by hexaammineruthenium (II) ion. J. Am. Chem. Soc. 96:940-492.

203 Hodges, H.L., Holwerda, R.A., and Gray, H.B. (1974). Kinetic studies of the reduction of ferricytochrome c by Fe(EDTA)2. J. Am. Chem. Soc. 96:3132-3137.

204 McArdle, J.V., Gray, H.B., Creutz, C., and Sutin, N. (1974). Kinetic studies of the oxidation of ferrocytochrome c from horse heart and Candida krusei by tris (1,10 phenathroline) cobalt(III). J. Am. Chem. Soc. 96:5737-5741.

205 Holwerda, R.A., Knoff, D.B., Gray, H. B., Clemmer, J. D., Crowiey, R., Smith, J.M., and Mauk, A.G. (1980). Comparison of the electron-transfer reactivities of tris(oxalato) cobalt(III) (Co(ox)33+) and tris (1,10 phenathroline) cobalt(III) (Co(phen)33+) with metalloproteins. J. Am. Chem. Soc. 102:1142-1146.

206 Eyring, H. (1935). The activated complex in chemical reaction. J. Chem. Phys. 3:107.

207 Marcus, R.A. (1956). On the theory of oxidation-reduction reactions involving electron transfer. I. J. Phys. Chem. 24:966-978.

208 Davison, A.J., Hamilton, R.T., and Kaminsky, L.S. (1971). A thermodynamic comparison of some oxidations of ferrocytochrome c. FEBS Lett. 19:19-26.

209 Hulett, L.G. (1968). The Reaction between Ferrocytochrome c and Hydrogen Peroxide. University of Cape Town, Cape Town, South Africa. Masters Thesis.

210 Radi, R., Thomson, L., Rubbo, H., and Prodanov, E. (1991). Cytochrome c-catalyzed oxidation of organic molecules by hydrogen peroxide. Arch Biochem Biophys. 288:112-117.

211 Radi, R., Bush, K.M., and Freeman, B.A. (1993). The role of cytochrome c and mitochondrial catalase in hydroperoxide-induced heart mitochondrial lipid peroxidation. Arch. Biochem. Biophys. 300:409-415.

212 Mochan, E., and Degn, H. (1969). Autocatalytic peroxidation of ferrocytochrome c. Biochim. Biophys. Acta 189:354-359.

213 Paleus, S., Ehrenberg, A., and Tuppy, A. (1955). Study of a peptic degradation product of cytochrome c. II: Investigation of the linkage between peptide moiety and prosthetic group. Acta Chem. Scand. 9:365-374.

214 Tu, A.T., Reinosa, J. A., and Hsiao, Y.Y. (1968). Peroxidative activity of hemepeptides from horse heart cytochrome c. Experientia 24:219-221.

215 The Worthington Enzyme Manual. (1972). Worthington Biochemical Corporation, New Jersey, USA, pp. 42-45.

216 Chan, E. (1986). Peroxidatic and Spectrophotometeric Characteristics of a 65-Residue Heme Peptide Derivative from Cytochrome c. University of British Columbia, Vancouver, B.C., Canada. Masters Thesis.

217 Wyllie, A.H. (1980). Cell death: The significance of apoptosis. Int. Rev. Cytol. 68:251-306.

218 Kontogeorgos, G., and Kovacs, K. (1995). Apoptosis in endocrine glands. Endocr. Pathol. 6:257-265.

219 Steller, H. (1995). Mechanism and genes of cellular suicide. Science 267:1445-1449.

220 Cohen, J.J. (1993). Apoptosis. Immunol. Today 14:120-130.

221 White, E. (1996). Life, death, and the pursuit of apoptosis. Genes Dev. 10:1-15.

222 Fisher, D.E. (2001). Pathways of apoptosis and the modulation of cell death in cancer. Hematol. Oncol. Clin. North. Am. 15:931-956.

223 Hayashi, T., and Faustman, D.L. (2001). Implications of altered apoptosis in diabetes mellitus and autoimmune disease. Apoptosis. 6:31-45.

224 Zou, H., Li, Y., Liu, X., and Wang, X. (1999). An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J. Biol. Chem. 274:11549-11556.

225 Green, D.R., and Reed, J.C. (1998). Mitochondria and apoptosis. Science 281:1309-1312.

226 Varkey, J., Chen, P., Jemmerson, R., and Abrams, J.M. (1999). Altered cytochrome c display precedes apoptotic cell death in Drosophila. J. Cell Biol. 144:701-710.

227 Kroemer, G., Zamzami, N., and Susin, S. A. (1997). Mitochondrial control of apoptosis. Immunol. Today 18:44-51.

228 Cai, J., Yang, J., and Jones, D.P. (1998). Mitochondrial control of apoptosis: the role of cytochrome c. Biochim. Biophys. Acta. 1366:139-149.

229 Zamzami, N., Kroemer, G. (2001). The mitochondrion in apoptosis: how Pandora's box opens. Nat. Rev. Mol. Cell. Biol. 2:67-71.

230 Zarotti, M., and Szabo, I. (1995). The mitochondrial permeability transition. Biochim. Biophys. Acta 1241:139-179.

231 Bernardi, P., and Petronilli, V. (1996). The permeability transition pore as a mitochondrial calcium release channel; a critical appraisal. J. Bioenerg. Biomembr. 28:129-136.

232 Kroemer, G. (1997). Mitochondrial implication in apoptosis. Towards an endosymbiont hypothesis of apoptosis evolution. Cell Death Differ. 4:443-456.

233 Marzo, I., Brenner, C., Zamzami, N., Jurgensmeier, J. M., Susin, S. A., Vieira, H. L., Prevost, M.C., Xie, Z., Matsuyama, S., Reed, J.C., and Kroemer, G. (1998). Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science 281:2027-2031.

234 Heiskanen, K.M., Bhat, M.B., Wang, H.W., Ma, J., Nieminen, A.L. (1999). Mitochondrial depolarization accompanies cytochrome c release during apoptosis in PC6 cells. J. Biol. Chem. 274:5654-5658.

235 Kroemer, G., Zamzami, N., and Susin, S. A. (1997). Mitochondrial control of apoptosis. Immunol. Today 86:147-157.

236 Bossy-Wetzel, E., Newmeyer, D.D., and Green, D.R. (1998). Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. EMBO J. 17:37-49.

237 Krohn, A.J., Wahlbrink, T., and Prehn, J.H.M. (1999). Mitochondrial depolarization is not required for neuronal apoptosis. J. Neurosci.19:7394-7404.

238 Kluck, R.M., Bossy-Wetzel, E., Green, D.R., and Newmeyer, D.D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275:1132-1136.

239 Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.I., Jones, D.P., and Wang, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275: 1129-1132.

240 Jurgensmeier, J.M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., and Reed, J.C. (1998). Bax directly induces release of cytochrome c from isolated mitochondria. Proc. Natl. Acad. Sci. U. S. A. 95: 4997-5002.

241 Li, H., Zhu, H., Xu, C.J., and Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94: 491-501

242 Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94: 481-490.

243 Schlesinger, P.H., Gross, A., Yin, X.M., Yamamoto, K., Saito, M., Waksman, G., and Korsmeyer, S.J. (1997). Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. Proc. Natl. Acad. Sci. U. S. A. 94:11357-11362.

244 Eskes, R., Antonsson, B., Osen-Sand, A., Montessuit, S., Richter, C., Sadoul, R., Mazzei, G., Nichols, A., and Martinou, J.C. (1998). Bax-induced cytochrome c release from mitochondria is independent of the permeability transition pore but highly dependent on Mg2+ ions. J. Cell Biol. 143:217-224.

245 Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J.J., Mazzei, G., Maundrell, K., Gambale, F., Sadoul, R., and Martinou, J.C. (1997). Inhibition of Bax channel-forming activity by Bcl-2. Science 277:370-372.

246 Shimizu, S., Narita, M., and Tsujimoto, Y., (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 399:483-487.

247 Basanez, G., Nechushtan, A., Drozhinin, O., Chanturiya, A., Choe, E., Tutt, S., Wood, K.A., Hsu, Y., Zimmerberg. J., and Youle, R.J. (1999). Bax, but not Bcl-xL, decreases the lifetime of planar phospholipid bilayer membranes at subnanomolar concentrations. Proc. Natl. Acad. Sci. U. S. A. 96:5492-5497

248 Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J.C. (1999). Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J. Cell Biol. 144:891-901.

249 Rosse, T., Olivier, R., Monney, L., Rager, M., Conus, S., Fellay, I., Jansen, B., and Bomer, C. (1998). Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. Nature 391:496-509.

250 Krajewski, S., Tanaka, S., Takayama, S., Schibler, M.J., Fenton, W., and Reed, J.C. (1993). Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res. 53:4701-4714.

251 Yang, E. and Korsmeyer, S.J. (1996). Molecular thanatopsis: a discourse on the Bcl-2 family and cell death. Blood 2:386-401.

252 Kim, C.N., Wang, X., Huang, Y., Ibrado, A.M., Liu, L, Fang, G. and Bhalla, K. (1997). Overexpression of Bcl-xL inhibits Ara-C-induced mitochondrial loss of cytochrome c and other perturbations that activate the molecular cascade of apoptosis. Cancer Res. 57:3115-3120.

253 Kroemer, G. (1997). The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nature Med. 3:614-620.

254 Reed, J.C. (1997). Double identity for proteins of the Bcl-2 family. Nature 387:773-776.

255 Reed, J.C. (1998). Bcl-2 family proteins. Oncogene 17:3225-3236.

256 Reed, J.C., Jurgensmeier, J.M., and Matsuyama, S. (1998). Bcl-2 family proteins and mitochondria. Biochim Biophys Acta 1366:127-137.

257 Wang, H.G., Rapp, U.R. and Reed, J.C. (1996). Bcl-2 targets the protein kinase Raf-1 to mitochondria. Cell 87:589-592

258 Chinnaiyan, A.M., O'Rourke, K., Lane, B.R., and Dixit, V.M. (1997). Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. Science 275:1122-1126.

259 James, C., Gschmeissner, S., Fraser, A., and Evan, G.I. (1997). CED-4 induces chromatin condensation in Schizosaccharomyces pombe and is inhibited by direct physical association with CED-9. Curr. Biol. 7:246-252.

260 Spector, M.S., Desnoyers, S., Hoeppner, D.J., and Hengartner, M.O. (1997). Interaction between the C.elegans cell-death regulators CED-9 and CED-4. Nature 385:653-656.

261 Wu, D., Wallen, H.D. and Nunez, G. (1997). Interaction and regulation of subcelluar localization of CED-4 by CED-9. Science 275:1126-1129.

262 Cook, S.A., Sugden, P.H., and Clerk, A. (1999). Regulation of Bcl-2 family proteins during development and in response to oxidative stress in cardiac myocytes: association with changes in mitochondrial membrane potential. Circ. Res. 85:940–949.

263 Skulachev, V.P. (1998). Cytochrome c in the apoptotic and antioxidant cascades. FEBS Lett. 423:275-280.

264 Petrosillo, G., Ruggiero, F.M., Pistolese, M., and Paradies, G. (2001). Reactive oxygen species generated from the mitochondrial electron transport chain induce cytochrome c dissociation from beef-heart submitochondrial particles via cardiolipin peroxidation. Possible role in the apoptosis. FEBS Lett. 509:435-438.

265 Turrens, J.F., Alexandre, A., and Lehninger, A.L. (1985). Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. Arch. Biochem. Biophys. 237:408-414.

266 Barja, G. (1999). Mitochondrial oxygen radical generation and leak: sites of production in states 4 and 3, organ specificity, and relation to aging and longevity. J. Bioenerg. Biomembr. 31:347-366.

267 Cadenas, E., and Davies, K.J. (2000). Mitochondrial free radical generation, oxidative stress, and aging. Free Radic. Biol. Med. 29:222-230.

268 Richter, C., Gogvadze, V., Laffranchi, R., Schlapbach, R., Schweizer, M., Suter, M., Walter, P., and Yaffee, M. (1995). Oxidants in mitochondria: from physiology to diseases. Biochim. Biophys. Acta 1271:67-74.

269 Loschen, G., Azzi, A., Richter, C., and Flohe, L. (1974). Superoxide radicals as precursors of mitochondrial hydrogen peroxide. FEBS Lett. 42:68-72.

270 Thannickal, V.J., and Fanburg, B.L. (2000). Reactive oxygen species in cell signaling. Am. J.Physiol. Lung Cell. Mol. Physiol. 279:L1005-L1028.

271 Cadenas, E. (1989). Biochemistry of oxygen toxicity. Ann. Rev. Biochem. 58:79-110.

272 Halliwell, B., and Gutteridge, M.C. (1990). Role of free radicals and catalytic metal ions in human disease: an overview. Methods in Enzymology 186:1-85.

273 Parke, D.V., and Sapota, A. (1996). Chemical toxicity and reactive oxygen species. Int. J. Occup. Med. Environ. Health 9:331-340.

274 Moslen, M.T. (1994). Reactive oxygen species in normal physiology, cell injury and phagocytosis. Adv. Exp. Med. Biol. 366:17-27.

275 Matsura, T., Kai, M., Fujii, Y., Ito, H., and Yamada, K. (1999). Hydrogen peroxide-induced apoptosis in HL-60 cells requires caspase-3 activation. Free Radic. Res. 30:73-83.

276 Lennon, S.V., Martin, S.J., and Cotter, T.G. (1991). Dose-dependent induction of apoptosis in human tumor cell lines by widely diverging stimuli. Cell Prolif. 24:203-214.

277 Chance, B., Sies, H., and Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. Physiol. Rev. 59:527-605.

278 Boveris, A., Oshino, N., and Chance, B. (1972). The cellular production of hydrogen peroxide. Biochem. J. 128:617-630.

279 Boveris, A., and Chance, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem. J. 134:707-716.

280 Boveris, A., and Chance, B. (1974). Optimal rates of hydrogen peroxide production in hyperbaric oxygen. In: Alcohol and Aldehyde Metabolizing Systems (R.G. Thurman, T., Yonetani, J.R. Williamson, and B. Chance, eds.) pp207-214, Academic press, New York and London.

281 Forman, H.J., and Boveris, A. (1982). Superoxide radical and hydrogen peroxide in mitochondria. In: Free Radicals in Biology (W.A. Pryor, ed.) vol V:65-90, Academic Press.

282 Freeman, B.A., and Crapo, J.D. (1981). Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. J. Biol. Chem. 256:10986-10992.

283 Loschen, G., Azzi, A., and Flohe, L. (1974). Mitochondrial hydrogen peroxide formation. In: Alcohol and Aldehyde Metabolising Systems (R.G. Thurman, T. Yonetani, J.R. Williamson, and B. Chance, eds.) pp215-229, Academic press, New York and London.

284 Wierusz-Wysocka, B., Wysocki, H., Byks, H., Zoxulinska, D., Wykretowicz, A., and Kazmierczak, M. (1995). Metabolic control quality and free radical activity in diabetic patients. Diabetes Res. and Clin. Pract. 27:193-197.

285 Hyslop, P.A., Hinshaw, D.B., Scraufstatter, I.U., Cochrane, C.G., Kunz, S., and Vosbeck, K. (1995). Hydrogen peroxide as a potent bacteriostatic antibiotic: Implications for host defense. Free Radic. Biol. Med. 19:31-37.

286 Yamada, T., and Ohyama, H. (1988). Radiation-induced interphase death of rat thymocytes is internally programmed (apoptosis). Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 53:65-75.

287 Lui, D. (1996). The roles of free radicals in amyotrophic lateral sclerosis. J. Mol. Neuroscience 7:159-167.

288 Miller, D.M., Buettner, G.R., and Aust, S.D. (1990). Transition metals as catalysts of "autoxidation" reactions. Free Radic. Biol. Med. 8:95-108.

289 Slater, A.F., Stefan, C., Nobel, I., van den Dobbelsteen, D.J., and Orrenius, S. (1995). Signalling mechanisms and oxidative stress in apoptosis. Toxicol. Lett. 82-83:149-153.

290 Halliwell, B., and Gutteridge, J.M. (1986). Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. Arch. Biochem. Biophys. 246:501-514.

291 Korsmeyer, S.J., Yin, X.M., Oltvai, Z.N., Veis-Novack, D.J., and Linette, G.P. (1995). Reactive oxygen species and the regulation of cell death by the Bcl-2 gene family. Biochim. Biophys. Acta 1271:63-66.

292 Coultas L, Strasser A. (2000). The molecular control of DNA damage-induced cell death. Apoptosis 5:491-507.

293 Pan, Z., Voehringer, D.W., and Meyn, R. E. (1999). Analysis of redox regulation of cytochrome c-induced apoptosis in a cell-free system. Cell Death Differ. 6:683-688.

294 Simizu, S., Imoto, M., Masuda, N., Takada, M., and Umezawa, K. (1996). Involvement of hydrogen peroxide production in erbstatin-induced apoptosis in human small cell lung carcinoma cells. Cancer Res. 56:4978-4982.

295 Nakamura, H., Nakamura, K., and Yodoi, J. (1997). Redox regulation of cellular activation. Ann. Rev. Immunol. 15:351-369.

296 Pierce, G.B., Parchment, R.E., and Lewellyn, A.L. (1991). Hydrogen peroxide as a mediator of programmed cell death in the blastocyst. Differentiation 46:181-186.

297 Kanno, S., Ishikawa, M., Takayanagi, M., Takayanagi, Y., and Sasaki, K. (1999). Exposure to hydrogen peroxide induces cell death via apoptosis in primary cultured mouse hepatocytes. Biol. Pharm. Bull. 2:1296-1300.

298 Wei, T., Ni, Y., Hou, J., Chen, C., Zhao, B., and Xin, W. (2000). Hydrogen peroxide-induced oxidative damage and apoptosis in cerebellar granule cells: protection by Ginkgo biloba extract. Pharmacol. Res. 41:427-433.

299 Suzuki, Y.S., Forman, H.J., and Sevanian, A. (1997). Oxidants as stimulators of signal transduction. Free Radic. Biol. Med. 22: 269-285.

300 Gate, L., Paul. J., Ba, G.N., Tew, K.D., and Tapiero, H. (1999). Oxidative stress induced in pathologies: the role of antioxidants. Biomed. Pharmacother. 53:169-180.

301 Lander, H.M. (1997). An essential role for free radicals and derived species in signal transduction. FASEB J. 11:118-124.

302 Rhee, S.G. (1999). Redox signaling: hydrogen peroxide as intracellular messenger. Exp. Mol. Med. 31:53-59.

303 Sundaresan, M., Yu, Z.X., Ferrans, V.J., Irani, K., and Finkel, T. (1995). Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. Science 270:296-299.

304 Sato, N., Iwata, S., Nakamura, K., Hori, T., Mori, K., and Yodoi, J. (1995). Thiol-mediated redox regulation of apoptosis. Possible roles of cellular thiols other than glutathione in T cell apoptosis. J. Immunol. 154:3194-3203.

305 Sun, X., and Ross, D. (1996). Quinone-induced apoptosis in human colon adenocarcinoma cells via DT-diaphorase mediated bioactivation. Chem. Biol. Interact. 100:267-276.

306 Kogure, K., Morita, M., Nakashima, S., Hama, S., Tokumura, A., and Fukuzawa, K. (2001). Superoxide is responsible for apoptosis in rat vascular smooth muscle cells induced by alpha-tocopheryl hemisuccinate. Biochim. Biophys. Acta 1528:25-30.

307 Jabs, T., Dietrich, R.A., and Dangl, J.L. (1996). Initiation of runaway cell death in an Arabidopsis mutant by extracellular superoxide. Science 273:1853-1856.

308 Li, P.F., Dietz, R., and von Harsdorf, R. (1999). Superoxide induces apoptosis in cardiomyocytes, but proliferation and expression of transforming growth factor-beta1 in cardiac fibroblasts. FEBS Lett. 448:206-210.

309 von Harsdorf, R., Li, P.F., and Dietz, R. (1999). Signaling pathways in reactive oxygen species-induced cardiomyocyte apoptosis. Circulation 99:2934-2941.

310 Buttke, T.M., and Sandstrom, P.A. (1994). Oxidative stress as a mediator of apoptosis. Immunol. Today 15:7-10.

311 Clutton, S. (1997). The importance of oxidative stress in apoptosis. Br. Med. Bull. 3:662-668.

312 Kowaltowski, A.J., and Vercesi, A.E. (1999). Mitochondrial damage induced by conditions of oxidative stress. Free Radic. Biol. Med. 26:463-471.

313 Shen, H.M., Yang, C.F., Ding, W.X., Liu, J., and Ong, C.N. (2001). Superoxide radical-initiated apoptotic signalling pathway in selenite-treated HepG(2) cells: mitochondria serve as the main target. Free Radic. Biol. Med. 30:9-21.

314 Troy, C.M., Derossi, D., Prochiantz, A., Greene, L.A., and Shelanski, M.L. (1996). Downregulation of Cu/Zn superoxide dismutase leads to cell death via the nitric oxide-peroxynitrite pathway. J. Neurosci. 16:253-261.

315 Troy, C.M., and Shelanski, M.L. (1994). Down-regulation of copper/zinc superoxide dismutase causes apoptotic death in PC12 neuronal cells. Proc. Natl. Acad. Sci. U. S. A. 91:6384-6387.

316 Manna, S.K., Zhang, H.J., Yan, T., Oberley, L.W., and Aggarwal, B.B. (1998). Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. J. Biol. Chem. 273:13245-13254.

317 Kiningham, K.K., Oberley, T.D., Lin, S., Mattingly, C.A., and St Clair, D.K. (1999). Overexpression of manganese superoxide dismutase protects against mitochondrial-initiated poly(ADP-ribose) polymerase-mediated cell death. FASEB J. 13:1601-1610.

318 Clemet, M., and Stamemkovic, I. (1996). Superoxide anion is a natural inhibitor of Fas-mediated cell death. EMBO J. 15:216-225.

319 Li, P.F., Dietz, R., and von Harsdorf, R. (1997). Differential effect of hydrogen peroxide and superoxide anion on apoptosis and proliferation of vascular smooth muscle cells. Circulation 96:3602-3609.

320 Pervaiz, S., Ramalingam, J.K., Hirpara, J.L., and Clement, M.V. (1999). Superoxide anion inhibits drug-induced tumor cell death. FEBS Lett. 459:343-348.

321 Samali, A., Nordgren, H., Zhivotovsky, B., Peterson, E., and Orrenius, S. (1999). A comparative study of apoptosis and necrosis in HepG2 cells: oxidant-induced caspase inactivation leads to necrosis. Biochem. Biophys. Res.Commun. 255:6-11.

322 von Knethen, A., Callsen, D., and Brune, B. (1999). Superoxide attenuates macrophage apoptosis by NF-kappa B and AP-1 activation that promotes cyclooxygenase-2 expression. J. Immunol. 163:2858-2866.

323 Buttke, T.M., and Sandstrom, P.A. (1995). Redox regulation of programmed cell death in lymphocytes. Free Radic. Res. 22:389-297.

324 Slater, A.F., Nobel, C.S., and Orrenius, S. (1995). The role of intracellular oxidants in apoptosis. Biochim. Biophys. Acta 1271:59-62.

325 Jacobson, M.D. (1996). Reactive oxygen species and programmed cell death. TIBS 21:83-86.

326 Cai, J., and Jones, D.P. (1998). Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss. J. Biol. Chem. 273:11401-11404.

327 Luetjens, C.M., Bui, N.T., Sengpiel, B., Münstermann, G., Poppe, M., Krohn, A,J., Bauerbach, E., Krieglstein. J., and Prehn, J.H.M. (2000). Delayed mitochondrial dysfunction in excitotoxic neuron death: cytochrome c release and a secondary increase in superoxide production. J. Neurosci. 20:5715-5723.

328 Esteve, J.M., Mompo, J., Garcia de la Asuncion, J., Sastre, J., Asensi, M., Boix, J., Vina, J.R., Vina, J., and Pallardo, F.V. (1999). Oxidative damage to mitochondrial DNA and glutathione oxidation in apoptosis: studies in vivo and in vitro. FASEB J. 13:1055-1064.

329 Macho, A., Hirsch, T., Marzo, I., Marchetti, P., Dallaporta, B., Susin, S.A., Zamzami N., and Kroemer, G. (1997). Glutathione depletion is an early and calcium elevation is a late event of thymocyte apoptosis. J. Immunol. 158:4612-4619.

330 Tan, S., Sagara, Y., Liu, Y., Maher, P., and Schubert, D. (1998). The regulation of reactive oxygen species production during programmed cell death. J. Cell Biol. 141:1423-1432.

331 Viña, J. (1990). Glutathione: Metabolism and Physiological Functions. CRC Press, Boca Raton, Florida

332 Ghibelli, L., Coppola, S., Fanelli, C., Rotilio, G., Civitareale, P., Scovassi, A.I., and Ciriolo, M.R. (1999). Glutathione depletion causes cytochrome c release even in the absence of cell commitment to apoptosis. FASEB J. 13:2031-2036.

333 Pereira, C., Santos, M.S., and Oliveira, C. (1999). Involvement of oxidative stress on the impairment of energy metabolism induced by A beta peptides on PC12 cells: protection by antioxidants. Neurobiol. Dis. 6:209–219.

334 Yatin, S.M., Aksenov, M., and Butterfield, D.A. (1999). The antioxidant vitamin E modulates amyloid beta-peptide-induced creatine kinase activity inhibition and increased protein oxidation: Implications for the free radical hypothesis of Alzheimer's disease. Neurochem. Res. 24:427–435.

335 Iacovitti, L., Stull, N.D., and Mishizen, A. (1999). Neurotransmitters, KCl and antioxidants rescue striatal neurons from apoptotic cell death in culture. Brain Res. 816:276–285.

336 Rössig, L., Hoffmann, J., Hugel, B., Mallat, Z., Haase, A., Freyssinet, J., Tedgui, A., Aicher, A., Zeiher, A. M., and Dimmeler, S. (2001). Vitamin C inhibits endothelial cell apoptosis in congestive heart failure. Circulation 104: 2182-2187.

337 Holguin, F., Moss, I., Brown, L.A., and Guidot, D.M. (1998). Chronic ethanol ingestion impairs alveolar type II cell glutathione homeostasis and function and predisposes to endotoxin-mediated acute edematous lung injury in rats. J. Clin. Invest. 101:761-768.

338 Mayer, M., and Noble, M. (1994). N-acetyl-L-cysteine is a pluripotent protector against cell death and enhancer of trophic factor-mediated cell survival in vitro. Proc. Natl. Acad. Sci. U. S. A. 91:7496-7500.

339 Iwata, S., Hori, T., Sata, N., Hirota, K., Sasada, T., Mitsui, A., Hirakawa, T., and Yodoi, J. (1997). Adult T cell leukemia (ATL)-derived factor/human thioredoxin prevents apoptosis of lymphoid cells induced by L-cystine and glutathione depletion: possible involvement of thiol-mediated redox regulation in apoptosis caused by pro-oxidant state. J. Immunol. 158:3108-3117.

340 Wong, G.H., Elwell, J.H., Oberley, L.W., and Goeddel, D.V. (1989). Manganous superoxide dismutase is essential for cellular resistance to cytotoxicity of tumor necrosis factor. Cell 58:923-931.

341 Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.L., and Korsmeyer, S.J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 75:241-251.

342 Longo, V.D., Ellerby, L.M., Bredesen, D.E., Valentine, J.S., and Gralla, E.B. (1997). Human Bcl-2 reverses survival defects in yeast lacking superoxide dismutase and delays death of wild-type yeast. J. Cell Biol. 37:1581-1588.

343 Esposti, M.D., Hatzinisiriou, I., McLennan, H., and Ralph, S. (1999). Bcl-2 and mitochondrial oxygen radicals. New approaches with reactive oxygen species-sensitive probes. J. Biol. Chem. 274:29831-29837.

344 Veis, D.J., Sorenson, C.M., Shutter, J.R., and Korsmeyer, S.J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75:229-240.

345 Korsmeyer,S.J., Shutter, J.R., Veis, D.J., Merry, D.E., and Oltvai, Z.N. (1993). Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death. Semin Cancer Biol. 6:327-332.

346 Voehringer, D.W., McConkey, D.J., McDonnell, T.J., Brisbay, S., and Meyn, R.E. (1998). Bcl-2 expression causes redistribution of glutathione to the nucleus. Proc. Natl. Acad. Sci. U. S. A. 95:2956-2960.

347 Mirkovic, N., Voehringer, D.W., Story, M.D., McConkey, D.J., McDonnell, T.J., and Meyn, R.E. (1997). Resistance to radiation-induced apoptosis in Bcl-2-expressing cells is reversed by depleting cellular thiols. Oncogene 15:1461-1470.

348 Kane, D.J., Sarafian, T.A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J.S., Ord, T., and Bredesen, D.E. (1993). Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. Science 262:1274-1277.

349 Schuler, M., and Green, D.R. (2001). Mechanisms of p53-dependent apoptosis. Biochem. Soc. Trans. 29:684-868.

350 Forman, H.J., Evans, H.J., Hill, R.L., and Fridovich I. (1973). Histidine at the active site of superoxide dismutase. Biochemistry 12:823-827.

351 O'Brien, P.J. (1966). Effect of hydrogen peroxide on cytochrome c. Biochem. J. 100:68P.

352 Hensley, K., Floyd, R.A., Gordon, B., Mou, S., Pye, Q.N., Stewart, C., West, M., and Williamson, K. (2002). Temporal patterns of cytokine and apoptosis-related gene expression in spinal cords of the G93A-SOD1 mouse model of amyotrophic lateral sclerosis. J. Neurochem. 82:365-374.

353 Beckman, J.S., Estevez, A.G., Crow, J.P., and Barbeito, L. (2001). Superoxide dismutase and the death of motoneurons in ALS. Trends Neurosci. 24:S15-20.

354 Williamson, K.S., Gabbita, S.P., Mou, S, West. M, Pye, Q.N., Markesbery, W.R., Cooney, R.V., Grammas, P., Reimann-Philipp, U., Floyd, R.A., and Hensley, K.

(2002). The nitration product 5-nitro-gamma-tocopherol is increased in the Alzheimer brain. Nitric Oxide. 6:221-227.

355 Hosler, B.A., Nicholson, G.A., Sapp, P.C., Chin, W., Orrell, R.W., de Belleroche, J.S., Esteban, J., Hayward, L.J., Mckenna-Yasek, D., Yeung, L., Cherryson, A.K., Dench, J.E., Wilton, SD., Laing. N.G., Horvitz, R.H., and Brown, R.H. Jr. (1996). Three novel mutations and two variants in the gene for Cu/Zn superoxide dismutase in familial amyotrophic lateral sclerosis. Neuromuscul. Disord. 6:361-366.

356 Banci, L., Bertini, I., Cabelli, D.E., Hallewell, R.A., Tung, J.W., and Viezzoli, M.S. (1991) A characterization of copper/zinc superoxide dismutase mutants at position 124. Zinc-deficient proteins. Eur. J. Biochem. 196:123-1288.

357 Hensley, K. (2002). Aging and neurogdegenerative diseases: Models and assessment of the impact and responses to ROS/RNS. Oxygen 2002 - Proceedings of the Oxygen Society meeting in San Antonio. Session 4:1-17.

358 Lyons, T.J., Nersissian, A., Huang, H., Yeom, H., Nishida, C.R., Graden, J.A., Gralla, E.B., and Valentine, J.S. (2000). The metal binding properties of the zinc site of yeast copper-zinc superoxide dismutase: implications for amyotrophic lateral sclerosis. J. Biol. Inorg. Chem. 5:189-203.

359 Nakamura, Y. Samejima, T., Kurihara, K., Tokjo, M., and Shibata, K. (1960). Peroxidase activity of hemoproteins. 2. Metmyoglobin and ctyochrome c. J. Biochem. Tokyo 48:862-869.

360 Flatmark, T. (1966). Studies on the peroxidase effect of cytochrome c. V. Spectrophotometric titration of a reversible conformational change of ferricytochrome c in acid aqueous solution. Acta Chem. Scand. 20:1470-1475.

361 Shashoua, V.E. (1965). Magneto-optical rotation spectroscope oxidation and reduction studies of cytochrome c. Arch. Biochem. Biophys. 111:550.

362 Dixon, M., and Webb, E. C. (1979). Enzymes, Third ed. Academic Press, New York.

363 Gopal, D., Wilson, G. S., Earl, R. A., and Casanovich, M. A.(1988). Cytochrome c: Ion binding and redox properties. J. Biol. Chem. 263:11652-11656.

364 McCord, J.M., and Day, E.D., Jr. (1978). Superoxide-dependent production of hydroxyl radical catalyzed by iron-EDTA complex. FEBS Lett. 86:139-142.

365 Tien, M., Morehouse, L.A., Bucher, J.R., and Aust, S.D. (1982). The multiple effects of ethylenediaminetetraacetate in several model lipid peroxidation systems. Arch. Biochem. Biophys. 218:450-458.

366 Baker, M.S., and Gebicki, J.M. (1984). The effect of pH on the conversion of superoxide to hydroxyl free radicals. Arch. Biochem. Biophys. 234:258-264.

367 Graf, E., Mahoney, J.R., Bryant, R.G., and Eaton, J.W. (1984). Iron-catalyzed hydroxyl radical formation. Stringent requirement for free iron coordination site. J. Biol. Chem. 259:3620-3624.

368 Koppenol, W.H. (1986). The reaction of ferrous EDTA with hydrogen peroxide: evidence against hydroxyl radical formation. J. Free Radic. Biol. Med. 1:281-284.

369 Koppenol, W.H., and Butler, J. (1984). The radiation chemistry of cytochrome c. Isr. J. Chem. 24:11-16.

370 Kolthoff, I.M., and Auerbach, C. (1952). Studies on the system iron-ethylenediamine tetraacetate. JACS 74:1452-1456.

371Murray, R.K., Granner, D.K., Mayes, P.A., and Rodwell, V.W. (2000). Harper's Biochemistry. 25th ed. Appletoon & Lange. Stamford, Connecticut.

372 Nicotera, P., Zhivotovsky, B., and OrreniusS. (1994). Nuclear calcium transport and the role of calcium in apoptosis. Cell Calcium 16:279-288.

373 Dowd, D.R. (1995). Calcium regulation of apoptosis. Adv. Second Messenger Phosphoprotein Res. 30:255-280.

374 McConkey, D.J., and Orrenius, S. (1997). The role of calcium in the regulation of apoptosis. Biochem. Biophys. Res. Commun. 239:357-366.

375 Appel, S.H., Beers, D., Siklos, L., Engelhardt, J.I., and Mosier, D.R. (2001). Calcium: the Darth Vader of ALS. Amyotroph Lateral Scler. Other Motor Neuron Disord. 2:S47-S54.

376 Appel, S.H., Beers, D., Smith, R.G., and Wilson, J.E. (2000). Altered calcium homeostasis in ALS as a target for therapy. Amyotroph Lateral Scler. Other Motor Neuron Disord. 1:27-32.

377 McConkey, D.J., Hartzell, P., Nicotera, P., and Orrenius, S. (1989). Calcium-activated DNA fragmentation kills immature thymocytes. FASEB J. 3:1843-1849.

378 Wyllie, A.H., Morris, R.G., Smith, A.L., and Dunlop, D. (1984). Chromatin cleavage in apoptosis: Association with condensed chromatin morphology and dependence on macromolecular synthesis. J. Pathol. 142:67-77.

379 Smith, C.A., Williams, G.T., Kingsdton, R., Jenkinson, E.J., and Owen, J.J.T. (1989). Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cell in thymic cultures. Nature 337:181-184.

380 Story, M.D., Stephens, L.C., Thomasovic. S.P., and Meyn, R.E. (1992). A role for calcium in regulating apoptosis in rat thymocytes irradiated in vitro. Int. J. Radiat. Biol. 61:243-251.

381 Cohen, J.J., and Duke, R.C. (1984). Glucocorticoid activation of calcium-dependent endonuclease in thymocyte nuclei leads to cell death. J. Immunol. 132:38-42.

382 Bray, T.M., and Bettger, W.J. (1990). The physiological role of zinc as an antioxidant. Free Radic. Biol. Med. 8:281-291.

383 Powell, S.R. (2000). The antioxidant properties of zinc. J. Nutr. 130(5S Suppl):1447S-1454S

384 Winston, G.W., Harvey, W., Beri, L., and Cedebaum, I. (1983). The generation of hydroxyl and alkoxyl radicals from the interaction of ferrous bipyridyl with peroxides. Biochem. J. 216:415-421.

385 Howell, R.R., and Wyngaarden, J.B. (1960). On the mechanism of peroxidation of uric acids by hemoproteins. J. Biol. Chem. 235:3544-3550.

386 Munday, R. (1988). Generation of superoxide radical, hydrogen peroxide and hydroxyl radical during the autoxidation of N,N,N',N'-tetramethyl-1,4-phenylenediamine. Chem. Biol. Interact. 65:133-143.

387 Marklund, S. (1980). Distribution of CuZn superoxide dismutase and Mn superoxide dismutase in human tissues and extracellular fluids. Acta Physiol. Scand. Suppl. 492:19-23.

388 Asada, K., Yoshikawa, K., Takahashi, M., Maeda, Y., and Enmanji, K. (1975). Superoxide dismutases from a blue-green alga, Plectonema boryanum. J. Biol. Chem. 250:2801-2807.

389 Buettner, G.R., and Jurkiewicz, B.A. (1996). Catalytic metals, ascorbate and free radicals: Combinations to avoid. Radiat. Res. 145:532-541.

390 Wells, W.W., and Xu, D.P. (1994). Dehydroascorbate reduction. J. Bioenerg. Biomembr. 26:369-377.

391 Halliwell, B. (1996). Vitamin C: antioxidant or pro-oxidant in vivo? Free Radic. Res. 25:439-454.

392 Zhou, S. (1995). Interactions between Ascorbate and Iron in DNA Damage. Simon Fraser University, Burnaby, B.C. Canada. Masters Thesis.

393 Ramirez-Tortosa, C., Andersen, O.M., Gardner, P.T., Morrice, P.C., Wood, S.G., Duthie, S.J., Collins, A.R., and Duthie, G.G. (2001). Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. Free Radic. Biol. Med. 31:1033-1037.

394 Wang, H., Cao, G.H., and Prior, R.L. (1997). Oxygen radical absorbing capacity of anthocyanins. J. Agr. Food. Chem. 45:304-309.

395 Tsuda, T., Shiga, K., Ohshima, K., Kawakishi, S., and Osawa, T. (1996). Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from Phaseolus vulgaris L. Biochem. Pharmacol. 52:1033-1039.

396 Forrest, V.J., Kang, Y.H., McClain, D.E., Robinson, D.H., and Ramakrishnan, N. (1994). Oxidative stress-induced apoptosis prevented by Trolox. Free Radic. Biol. Med. 16:675-684.

397 McClain, D.E., Kalinich, J.F., and Ramakrishnan, N. (1995). Trolox inhibits apoptosis in irradiated MOLT-4 lymphocytes. FASEB J. 9: 1345-1354.

398 Salgo, M.G., and Pryor, W.A. (1996). Trolox inhibits peroxynitrite-mediated oxidative stress and apoptosis in rat thymocytes. Arch. Biochem. Biophys. 333: 482-488.

399 Chen, H., and Tappel, A.L. (1995). Vitamin E, selenium, trolox C, ascorbic acid palmitate, acetylcysteine, coenzyme Q, beta-carotene, canthaxanthin, and (+)-catechin protect against oxidative damage to kidney, heart, lung and spleen. Free. Radic. Res. 22: 177-186.

400 Barr, D.P., Gunther, M.R., Deterding, L.J., Tomer, K.B., and Mason, R.P. (1996). ESR spin-trapping of a protein-derived tyrosyl radical from the reaction of cytochrome c with hydrogen peroxide. J. Biol. Chem. 271:15498-15503.

401 Grankvist, K., Marklund, S.L, and Taljedal, I.B. (1981). CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. Biochem. J. 199:393-839

402 Stridh, H., Kimland, M., Jones, D.P., Orrenius, S., and Hampton, M.B. (1998). Cytochrome c release and caspase activation in hydrogen peroxide- and tributyltin-induced apoptosis. FEBS Lett. 429:351-355.

403 Johnson, D.E. (2000).Noncaspase proteases in apoptosis. Leukemia. 14:1695-703.

404 Solary, E., Eymin, B., Droin, N., and Haugg, M. (1998). Proteases, proteolysis, and apoptosis. Cell Biol. Toxicol. 14:121-132.