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Physical and Catalytic Properties of a Peroxidase Derived from Cytochrome C

Johannes Everse, Chyong-Jy J. Liu and Penelope W. Coates

Cell Biology and Biochemistry
Texas Tech University Health Sciences Center
Lubbock, TX 79430

Corresponding Author:

Johannes Everse, Ph.D.
Cell Biology and Biochemistry
Texas Tech University Health Sciences Center
Lubbock, TX 79430
Tel: 806-743-2700 ext 234
Fax: 806-743-2990
E-mail: Johannes.everse@ttuhsc.edu

Abbreviations:

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; Cytochrome c_{px} : the peroxidase form of cytochrome c; DTNB: 5,5'-dithio-bis-2-nitrobenzoic acid; DTPA: diethylenetriaminepentaacetic acid; DOPA: 3,4-dihydroxyphenylalanine; GSH: reduced glutathione; MESNA: sodium 2-mercaptoethanesulfonate; PBS: phosphate-buffered saline.

ABSTRACT

Except for its redox properties, cytochrome c is an inert protein. However, dissociation of the bond between methionine-80 and the heme iron converts the cytochrome into a peroxidase. Dissociation is accomplished by subjecting the cytochrome to various conditions, including proteolysis and hydrogen peroxide (H₂O₂)-mediated oxidation.

In affected cells of various neurological diseases, including Parkinson's disease, cytochrome c is released from the mitochondrial membrane and enters the cytosol. In the cytosol cytochrome c is exposed to cellular proteases and to H₂O₂ produced by dysfunctional mitochondria and activated microglial cells. These could promote the formation of the peroxidase form of cytochrome c.

In this study we investigated the catalytic and cytolytic properties of the peroxidase form of cytochrome c. These properties are qualitatively similar to those of other heme-containing peroxidases. Dopamine as well as sulfhydryl group-containing metabolites, including reduced glutathione and coenzyme A, are readily oxidized in the presence of H₂O₂. This peroxidase also has cytolytic properties similar to myeloperoxidase, lactoperoxidase, and horseradish peroxidase. Cytolysis is inhibited by various reducing agents, including dopamine.

Our data show that the peroxidase form of cytochrome c has catalytic and cytolytic properties that could account for at least some of the damage that leads to neuronal death in the parkinsonian brain.

Research highlights

- Cytochrome c in which the methionine-iron bond is broken by proteolysis acts as a potent peroxidase.
- Proteolysis of released cytochrome c is likely to occur in the cytosol of Parkinsonian neurons.

- This peroxidase is able to oxidize metabolites including glutathione and coenzyme A.
- This peroxidase is also able to oxidize sulfhydryl groups in proteins leading to their inactivation.
- This peroxidase could account for most of the aberrant reactions occurring in Parkinsonian neurons.

Key words: cytochrome c; peroxidase; Parkinson's disease; neurodegeneration; cytolysis; cellular metabolites.

Running Title: Properties of Cytochrome C_{px}

1. INTRODUCTION

Parkinson's disease is a neurodegenerative disease, usually affecting older people. The disease is characterized by a selective loss of dopaminergic neurons and the development of Lewy bodies in the substantia nigra region of the brain. When symptoms first appear in patients, 60-70% of the neurons of the substantia nigra are already degenerated and the striatal dopamine level is reduced by 80% [1,2]. Patients are commonly treated with levo-DOPA, in an attempt to partly restore the dopamine that is lost during degeneration of the neurons.

Although many studies have been conducted with patients as well as with animal models attempting to inhibit the progression of the disease, successful results have been scarce, perhaps due to the fact that the cause of the disease is still obscure and many questions about the processes involved in the progression of the disorder are still left unanswered.

Based on a close examination of the aberrant products found in the substantia nigra of Parkinson patients and assuming that these products were generated by enzymatic action, we

concluded that these products appear to be the products of a heme peroxidase [3]. These enzymes are typically non-specific for their substrates to be oxidized, but are specific for hydrogen peroxide as the reducing substrate. Moreover, the oxidative energy available from the peroxidase reaction is about 50 kcal/mole, which is sufficient energy to oxidize proteins, lipids and nucleic acids as well as many other biological compounds [3].

Many cells contain peroxidases, but they are harmless because normally H_2O_2 is only present at very low concentrations *in vivo*. However, it is well established that in parkinsonian neurons dysfunctional mitochondria produce abnormally high amounts of H_2O_2 , often also referred to as reactive oxygen species (ROS) [4]. Hence, this H_2O_2 could trigger a peroxidase into oxidizing various important cellular components, eventually leading to neuronal death.

To test this hypothesis, we analyzed the substantia nigra of several dogs some time ago, and found peroxidase activity to be present in these tissues. We suggested at that time that the oxidative damage that occurs in affected neurons of Parkinson's disease may at least in part be catalyzed by a peroxidase [5]. Since that time peroxidase activity has also been observed in a rat brain homogenate [6] and in the substantia nigra of normal and parkinsonian human brain tissues [7,8]. Unfortunately, much effort notwithstanding, the identity of this endogenous peroxidase activity has not yet been firmly established [9].

A second well established fact associated with parkinsonism is the aberrant release of cytochrome c from the mitochondrial membrane [10,11]. This cytochrome c is known to be involved in triggering the apoptotic pathway [12,13]. However, cytosolic cytochrome c may also be involved in other reactions.

The fact that cytochrome c can form a peroxidase by proteolytic action has been known for about 50 years. Harbury and Loach [14] showed that extensive treatment of cytochrome c

with pepsin yields a heme moiety with as few as 8 to 20 amino acids remaining attached, one of which is the heme-bound histidine. These products are known as microperoxidases and are commercially available. These mini-enzymes have been extensively studied over the past 30 years (reviewed by [15]), and are known to not only catalyze oxidative reactions but also possess cytolytic activity [16].

Native human cytochrome c is a heme protein with a molecular weight of 12,384, containing 104 amino acid residues (Figure 1). The heme moiety is covalently bound to the protein by two cysteine residues, and ligands 5 and 6 of the heme iron are both occupied: ligand 5 is occupied by an imidazole nitrogen of histidine¹⁸, and ligand 6 is occupied by the sulfur atom of methionine⁸⁰. As such, it has no enzymatic activity because it has no substrate binding site. However, a dissociation of the Fe-Met⁸⁰ bond creates a free sixth ligand, that is able to bind H₂O₂ in a manner similar to other heme peroxidases. Hence, the only requirement for converting cytochrome c to a peroxidase is a dissociation of the bond between Met⁸⁰ and the heme iron.

This information led us to the hypothesis that cytochrome c may be involved in the early stages of Parkinson's disease and perhaps in other neurological diseases as well [3,17]. Cytochrome c, before or after its release from dysfunctional mitochondrial membranes may be converted to its peroxidase form by H₂O₂ or by cytosolic proteases and as such may at least in part be responsible for the oxidative and cytolytic reactions that are hallmarks of neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS).

In view of the potential role that the peroxidase form of cytochrome c may play in the process of neurodegeneration, we investigated the catalytic and cytolytic properties of this enzyme (hereinafter referred to as cytochrome c_{px} in order to prevent confusion with the bacterial

enzyme already known for decades as cytochrome c peroxidase). Our results indicate that cytochrome c_{px} does possess the catalytic properties required to catalyze the types of damage that are observed in parkinsonian brain cells.

2. MATERIALS AND METHODS

2.1. Materials

Cytochrome c and all other chemicals used in this study were purchased from Sigma-Aldrich Chemical Corp., St. Louis MO.

2.2. Methods

All experiments were carried out at least in triplicate. Graphs of the most representative results are shown in the figures.

2.2.1. Preparation of Cytochrome C_{px}

Cytochrome c (5.5 mg) was dissolved in 4.4 ml distilled water and the pH was adjusted to 2.6 with 1 N HCl. To the solution was added 10 :1 of a 1mg/ml pepsin solution and the mixture was incubated at room temperature for 60 min. The pH was then adjusted to 7.5 with a 0.2 M Na_2HPO_4 solution and the mixture was chromatographed over a Sephadex-25 column, equilibrated in distilled water, to remove the pepsin. The column was eluted with distilled water and the colored fractions were collected, dialyzed against 2 liters of distilled water overnight, and lyophilized. Mass spectrophotometric analyses were performed by the Center for Biotechnology and Genomics at Texas Tech University.

2.2.2. Assay for Peroxidase Activity

Peroxidase activity was measured by determining the rate of hydrogen peroxide-mediated oxidation of the commonly used substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) spectrophotometrically at 740 nm. Nine hundred μ l of a 9.1 mM solution of ABTS in 0.1 M Na-phosphate buffer, pH 5.5, was placed in a 1-ml cuvette, as well as 100 μ l 100 mM H_2O_2 in the same buffer. The reaction was started by the addition of 10 - 50 μ l of the solution to be tested, and assays were normally carried out for 3 minutes at room temperature. Measurements were made with a Hewlett-Packard UV/vis spectrophotometer Model 8450A, equipped with a Model HP-89100A temperature controller. Reference cuvettes contained the same ingredients as the sample cuvettes, except for the peroxidase. This was done to correct for any non-catalyzed oxidation of substrates.

Specific peroxidase activity is expressed as moles ABTS oxidized per mole of cytochrome c_{px} (MW = 12,384) per minute under the conditions of the assay. H_2O_2 concentrations were determined spectrophotometrically at 240 nm ($\epsilon = 43.6 M^{-1}.cm^{-1}$ [18,19]).

Dopamine oxidation was measured spectrophotometrically at 476 nm. A 1-ml cuvette contained 880 μ l 50 mM tris-HCl buffer, pH 7.2, containing 1 mM diethyltriaminepentaacetic acid (DTPA); 50 μ l cytochrome c_{px} (100 μ g/ml); and 20 μ l 40 mM dopamine. The reaction was started by the addition of 50 μ l 100 mM H_2O_2 .

2.2.3. Oxidation of Sulfhydryl Groups by SOD1 Peroxidase

Oxidation of sulfhydryl group-containing substances was determined by following the disappearance of sulfhydryl groups using the 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) method as described by Wright and Viola [20]. The sulfhydryl group-containing compound was dissolved in 10 ml 50 mM tris-HCl buffer, pH 7.2, containing 1 mM DTPA, to a final

concentration of 33 mM. Added were 50 :l cytochrome c_{px} (1 mg/ml). 0.9 ml was then transferred to a 1-ml cuvette and 50 :l 1 M tris buffer, pH 8.0, and 50 :l distilled water were added to the cuvette, followed by rapid mixing and recording the absorbance of the solution at 412 nm (value a). Another 0.9 ml aliquot was then transferred to another 1-ml cuvette and the experiment was repeated, except that 50 :l 30 mM DTNB solution in tris buffer, pH 8.0, was added instead of the water (value b). The net absorbance (value b-a) was considered the absorbance of 100% SH groups. To start the reaction, 50 :l of 1.0 M H_2O_2 were then added to the 10 ml reaction mixture and at the indicated times 0.9 ml aliquots were removed, treated as above with DTNB and the absorbance of the treated solution was determined (value c). The decrease in SH content was then determined by subtracting value c from value b. Percentage SH groups oxidized was then determined as $(b - c)/(b - a)$. All reactions and measurements were carried out at 37EC.

2.2.4. Assay for Cytolytic Activity

The assay to measure the rate of cytolysis, catalyzed by a peroxidase is described in detail elsewhere [21,22]. Briefly, cytolysis can be measured spectrophotometrically by measuring the decrease in turbidity that occurs when cells lyse. This decrease is most pronounced with erythrocytes, which do not contain nuclei, mitochondria, and other insoluble bodies that cause a remaining amount of turbidity and therefore yield much more “noisy” curves [23]. The decrease in turbidity can be conveniently measured at a wavelength where absorbance of all components in the cuvette is minimal. We have routinely used 600 nm for this purpose. It is a prerequisite that the cells be dispersed in an isotonic buffer in order to prevent the occurrence of non-catalyzed cell lysis. All cytolitic assays were performed at 37EC.

A typical assay for cytolitic activity is done in a 3-ml cuvette, which contains about 2×10^6 rabbit erythrocytes, $200 \mu\text{M}$ H_2O_2 , $25 \mu\text{M}$ potassium iodide and $100 \mu\text{g}$ cytochrome c_{px} in 9.5 mM Na-phosphate buffer, pH 7.5, containing 140 mM NaCl. Reactions were started with the addition of H_2O_2 . Reference cuvettes contained all ingredients, except the erythrocytes. Erythrocytes were separated from 1 - 2 ml freshly drawn rabbit blood, obtained via the ear vein. This procedure was approved by the Institutional Animal Care and Use committee.

3. RESULTS.

3.1. Assay for Peroxidase Activity

ABTS is one of the more sensitive substrates to measure peroxidase activity. The generation of oxidized ABTS by a peroxidase is usually followed spectrophotometrically at 410 nm . However, this is close to the Soret absorption band of cytochrome c , which makes ABTS less suitable to use as a substrate for measuring the peroxidase activity of cytochrome c . But oxidized ABTS has also a large absorption maximum above 700 nm , where the absorbance of cytochrome c is negligible (Figure 2). We made use of this property in the past when we measured the peroxidase activity of methemoglobin [24]. Accordingly, in the present study we used 740 nm as the wavelength for measuring the catalytic activity of cytochrome c_{px} . We determined the extinction coefficient of oxidized ABTS at 740 nm to be $23,480 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

3.2. Formation of Cytochrome C_{px} by Proteolysis

Pepsin has been the protease of choice to catalyze the conversion of cytochrome c to its peroxidase form *in vitro* on a preparative scale [25,26]. Figure 3 shows the generation of peroxidase activity as a function of time when cytochrome c is hydrolyzed with pepsin using the

procedure described in Methods. Maximum specific activity was obtained after a 90-min incubation. Pepsin-catalyzed hydrolysis will continue after 90 min but may cause some loss of peroxidase activity. Incubation of a cytochrome c solution for 20 hours in the absence of pepsin results in a spontaneous generation of a small amount of peroxidative activity, as indicated in Figure 3.

It should be mentioned that acidification of a cytochrome c solution in the absence of any proteolytic enzyme leads to about a 5-fold increase in peroxidase activity. This increase is completely reversed when the solution is subsequently neutralized again, even when the pH was adjusted to as low as 1.1. This may be caused by a partial and reversible unfolding of the cytochrome at low pH.

Mass spectroscopy results show that pepsin causes a hydrolysis of the cytochrome at Leu⁶⁴ and at Glu⁶⁶ (see Figure 1), yielding two peptides of 7675 and 7937 daltons, each having peroxidase activity, as well as two inert (and colorless) peptides of 4266 and 4583 daltons (Figure 4). Preparing cytochrome c_{px}, as described in Methods, yields a preparation that contains a mixture of the two heme-containing peptides. Such a preparation was used in the experiments described below.

A similar hydrolysis experiment in which trypsin was used as the hydrolytic enzyme at pH 7.2 did not result in the generation of any significant amount of peroxidase activity.

3.3. Oxidation of Various Metabolites and Antioxidants by Cytochrome C_{px}

As is characteristic for most heme-containing peroxidases, cytochrome c_{px} in the presence of H₂O₂ will oxidize a large variety of compounds. Besides ABTS, the compounds that can also serve as substrates include reduced glutathione, L-DOPA, dopamine, N-acetylcysteine,

minocycline, curcumin, 3-aminotyrosine, desferal, apomorphine, dimercaptopropanol, and clioquinol, among many others.

Coenzyme A is slowly oxidized to the disulfide form in the presence of low millimolar concentrations of H_2O_2 . This oxidation is significantly accelerated in the presence of cytochrome c_{px} , as shown in Figure 5. Thus, the presence of 2 :g cytochrome c_{px} causes a 5-fold acceleration in the rate of coenzyme A oxidation by 1 mM H_2O_2 .

3.4. Inhibition of Dopamine Oxidation by Various Antioxidants

Since it is well established at this point that the excess H_2O_2 , produced by dysfunctional mitochondria in parkinsonian brain cells as well as by activated microglial cells surrounding the affected neurons, is a participant in the neuronal degeneration, removal of this excess H_2O_2 may cause an attenuation of the degenerative process. This could be achieved by supplying the brain with antioxidant compounds that are more readily oxidized than cellular components such as dopamine. Accordingly, we tested a variety of antioxidants for their ability to inhibit the oxidation of 800 :M dopamine *in vitro*. These compounds included curcumin, caffeic acid, 3-aminotyrosine, minocycline, N-Ac-cysteine, cysteine, GSH, dimercaptopropanol, and Na-2-mercaptoethane-sulfonate (MESNA). Of these, cysteine, N-Ac-cysteine and GSH were oxidized much faster than dopamine, as shown by a lag time in the oxidation of dopamine (Figure 6). A 50% inhibition of the rate of 800 :M dopamine oxidation was observed with 150 :M 3-aminotyrosine, 80 :M MESNA, and 30 :M dimercaptoethanol. MESNA and dimercaptoethanol are sulfhydryl compounds commonly used as antidotes against lead and mercury poisoning. Caffeic acid at 0.5 mM as well as minocycline at 0.1 mM showed no inhibition. Curcumin was shown to have considerable beneficial effects toward both Parkinson's and Alzheimer's diseases

[27-30]. Inhibition of dopamine oxidation by curcumin is shown in Figure 7. Note that each of the tested compounds is also a substrate for cytochrome c_{px} ; therefore the observed inhibition is caused by a competition between dopamine and the compound tested for the active site of the peroxidase.

3.5. Inactivation of Cytochrome C_{px} by H_2O_2

In the absence of any substrate, cytochrome c_{px} is rapidly inactivated by relatively low concentrations of H_2O_2 . This appears to be a self-oxidation of the enzyme. This self-oxidation is also apparent during assays with some slow-oxidizing substrates, which show a significant decrease in activity with time. Thus, the oxidation of coenzyme A shows that after about 10 min the rate becomes similar to that of the uncatalyzed rate (Figure 5), suggesting that under these assay conditions it takes about 10 minutes for the cytochrome c_{px} to become inactivated. Addition of a second aliquot of the peroxidase to an assay mixture after the rate has decreased to that of the uncatalyzed reaction restores the initial rate, indicating that the decrease in rate is not caused by a decrease in substrate concentration (data not shown).

3.6. Cytolytic Properties of Cytochrome C_{px}

Cytochrome c_{px} in the presence of certain halides will promote the cytolysis of various cells in a reaction similar to that of myeloperoxidase [31,32], lactoperoxidase [23], horseradish peroxidase [22], and hemin [33]. A typical example is shown in Figure 8, illustrating the rate of cytolysis of rabbit erythrocytes at various concentrations of H_2O_2 . No lysis occurs in the absence of KI or in the absence of H_2O_2 . The rate of lysis is pH dependent and shows a rather sharp maximum at pH 7.5 (data not shown).

This lytic activity of cytochrome c_{px} is inhibited by a variety of compounds, including dopamine, reduced glutathione, apomorphine, desferal, 3-aminotyrosine, minocycline and L-DOPA. The most potent of these compounds was 3-aminotyrosine, which completely inhibited cell lysis at 5 μ M, followed by minocycline, which inhibited completely at 20 μ M. All other compounds required concentrations of at least 100 μ M to completely inhibit cytolysis. Inhibition by L-DOPA is shown in Figure 9.

4. DISCUSSION

4.1. Conversion of Cytochrome C to Cytochrome C_{px}

The conversion of cytochrome c to a peroxidase, using much less drastic conditions than the extensive proteolysis used by Harbury and Loach [14], has been observed for several years. Thus, treatment of cytochrome c with concentrations of H_2O_2 between 0.1 and 1 mM results in the generation of peroxidase activity [34,35]. Also, nitration of tyr⁶⁷ of the cytochrome leads to a large increase in its peroxidase activity [36]. Furthermore, Burkitt and his colleagues showed that in the presence of 250 μ M H_2O_2 cytochrome c becomes a compound with peroxidase activity, and these authors proposed that this form of cytochrome c may play an important role in redox signaling in apoptosis [4,13,37]. Kagan and co-workers [38] showed that cytochrome c binds avidly to phosphatidylserine, yielding a complex with potent peroxidase activity that readily catalyzes the peroxidation of lipids. Following release from the mitochondrial inner membrane, cytochrome c can also bind to the cardiolipin that is present in the inner membrane. Kagan and colleagues performed detailed studies on the structural and biochemical properties of the cytochrome c/cardiolipin complex. This complex has potent peroxidase activity and promotes the oxidation of the cardiolipin [39]. They subsequently showed that as a result of the binding to

cardiolipin, cytochrome c undergoes a conformational change that results in a dissociation of the Fe- methionine ligand [40,41], which explains the generation of peroxidase activity by the cardiolipin-bound cytochrome. Finally, Mizuta et al. [42] showed that in mouse fibroblasts stimulated with a combination of the calcium ionophore A23187 and arachidonic acid, cytosolic serine proteases not only caused the release of cytochrome c from the mitochondrial membrane but that these proteins are also somehow involved in causing cell death. The latter observation could indicate that the proteases converted the cytochrome to cytochrome c_{px} , which in turn caused cell death by means of its oxidative actions. If such a reaction should also occur in affected neurons, it is possible that cytochrome c, following proteolysis and now acting as a peroxidase, may at least be partly responsible for the oxidation of important metabolites (reduced glutathione, coenzyme A, dopamine, etc.) as well as of life-sustaining proteins and lipids.

4.2. Peroxidase Activity In Vivo

Peroxidases and pseudo-peroxidases are present in many cell types. Usually, these enzymes are inactive, because their essential substrate, H_2O_2 , is lacking. However, when an H_2O_2 generating system is activated, peroxidases will start exerting their oxidative actions. Such is the case, for example, in polymorphonuclear leukocytes, which are rich in myeloperoxidase.

Activation of these cells includes an activation of their NADPH oxidase, which initiates the generation of H_2O_2 . This in turn allows myeloperoxidase to exert its destructive action, leading to the cytolysis of any phagocytosed organism [43].

In vivo concentrations of H_2O_2 in brain tissues are not yet well established. This is largely due to the rapid influx and efflux of H_2O_2 through cellular and mitochondrial membranes. Catalase and glutathione peroxidase prevent a build-up of any H_2O_2 that may be formed by

cellular oxidases or by the mitochondrial electron transport chain under normal conditions. Steady state concentrations have been estimated to be as low as a few μM [44,45]; however, H_2O_2 concentrations up to $100 \mu\text{M}$ are generally considered to be physiological concentrations [54-57]. In parkinsonian brain tissues, however, this concentration could be considerably higher, considering the high rate at which activated microglia produce H_2O_2 [52,53]. In addition, the malfunctioning electron transport chain also produces an unknown amount of H_2O_2 . When the rate of H_2O_2 production exceeds the capacity of catalase and glutathione peroxidase to neutralize the H_2O_2 , any peroxidases that are present in the affected neurons, including any cytosolic cytochrome c_{px} , may become active under those conditions and cause oxidative damage to cellular components.

4.3. Consequences of Coenzyme A Oxidation

The fact that a depletion of reduced glutathione occurs in parkinsonian neurons is well established [46-48]. We found that oxidation of reduced glutathione is catalyzed by cytochrome c_{px} . *In vivo*, oxidized glutathione can be reduced again by glutathione reductase with the aid of NADPH, thus providing a mechanism for the regeneration of reduced glutathione. Our results show that coenzyme A is also oxidized by H_2O_2 , and the rate of oxidation is accelerated by cytochrome c_{px} . This indicates that this important cofactor is probably also subject to oxidation in parkinsonian neurons, albeit at a slower rate than GSH oxidation. However, mammals do not have a coenzyme A reductase. Therefore, maintenance of functional cellular coenzyme A levels will depend on either a disulfide transfer, which requires high levels of reduced glutathione, or biosynthesis of new coenzyme A, which requires the availability of a continuous supply of pantothenic acid (vitamin B5). Whether the levels of coenzyme A in affected brain cells are low

is currently not known, but indirect evidence suggests that they are. Since the brain is highly dependent on either the aerobic oxidation of glucose or ketone bodies as its energy sources, both of which require coenzyme A as an essential cofactor, it follows that a significant depletion of functional coenzyme A could be a contributing factor in the demise of affected neurons in parkinsonism.

A lack of sufficient amounts of coenzyme A would cause the brain to be more dependent on glycolysis for its ATP production, causing increased levels of brain lactate. Increased levels of lactate were indeed detected in both Parkinson patients and in animal models (49-51).

Furthermore, Parkinson's patients in general have low cholesterol levels and 3-hydroxy-3-methyl-glutaryl-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, was found to be reduced in Parkinson's patients [52,53]. Given the role of coenzyme A in sterol biosynthesis, a deficiency in coenzyme A could explain these observations.

Biochemical analyses of brains from Parkinson's patients also show decreased levels of phosphatidylethanolamine, phosphatidylcholine and polyunsaturated fatty acids [54-56]. Again, since coenzyme A is required for the biosynthesis of these compounds, such results may be expected in cases where a deficiency in coenzyme A exists.

Given these considerations, it is of considerable importance that studies be performed analyzing affected tissues for their coenzyme A content as well as for the possible presence of cytochrome c_{px} .

4.4. Inhibition of Dopamine Oxidation by Various Compounds

Several anti-oxidant compounds were shown to have an attenuating effect on the neurodegenerative progression in Parkinson's patients and/or animal models (e.g. [57,58]). If

neuronal damage during neurodegeneration is in part caused by the excessive production of H_2O_2 , then removal of the H_2O_2 before it can oxidize important cellular metabolites or enzymes should result in an attenuation of the progression of neurodegeneration. This can theoretically be accomplished by the administration of oxidizable compounds that have a lower K_m for the peroxidase than the cellular metabolites and would therefore be oxidized preferentially, thereby depleting excess H_2O_2 . To be effective *in vivo*, such compounds have to be able to pass the cell membrane as well as the blood-brain barrier, and the compound itself as well as its oxidation product(s) should be non-toxic. For this reason we investigated if and to what extent various relevant compounds inhibit the oxidation of dopamine by cytochrome c_{px} . Currently, L-DOPA is used as a drug in parkinsonism in an attempt to replenish the dopamine in neuronal cells. A different strategy may be to use drugs that are oxidized preferentially to dopamine, and may therefore result in a retention of dopamine levels in affected cells.

A considerable amount of research has shown a role of α -synuclein in vesicle trafficking and its toxic effects in parkinsonism (for a recent review, see [59]). Four tetrahydroquinolines were recently identified that protect against these toxic effects [60]. These compounds also protect against rotenone toxicity in rat midbrain neurons. The authors conclude that “these compounds must be acting on some deeply rooted biological process, linking α -synuclein toxicity, vesicle trafficking defects, and mitochondrial dysfunction” [59]. We propose that this process may be the oxidative actions of cytochrome c_{px} and H_2O_2 .

4.5. Cytolysis in Parkinson's Disease.

The cytolytic activity of cytochrome c_{px} indicates that this peroxidase is also capable of causing severe damage to membrane structures. The possibility that the cytolytic

action of certain peroxidases may also take part in the destructive mechanism of neurodegeneration has so far not been considered. It is known that various heme-containing peroxidases, as well as hemin itself, catalyze cell lysis in the presence of H_2O_2 and a halide ion [22,23,33,43]. If it can be shown that cytochrome c_{px} is present in parkinsonian neurons, then the possibility of cytolytic action participating in neurodegeneration should receive serious consideration.

4.6. Peroxidase Involvement in Other Neurodegenerative Diseases.

Citing experimental results we concluded earlier that the biochemical processes leading to neurodegeneration are very similar, if not the same in several neurological diseases, including Parkinson's and Alzheimer's diseases as well as ALS, and that the oxidative reactions are most likely catalyzed by a peroxidase [3]. Current information suggests that the identity of this peroxidase may not be the same among the various neurodegenerative diseases. Thus, the A β peptide is known to be intimately associated with Alzheimer's disease. This peptide was shown to bind heme with high affinity, and the product possesses peroxidase activity [61-63]. Similarly, it is well established that Cu,Zn-superoxide dismutase is intimately involved in ALS. This enzyme, when exposed to H_2O_2 , transforms into a peroxidase [64-66]. This peroxidase was recently shown to be present in the nuclei and cytosol of ALS transfected SH-SY5Y cells but not in normal SH-SY5Y cells [67]. These findings lend further support to our hypothesis that peroxidases may be intimately involved in neurodegeneration.

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ACCEPTED MANUSCRIPT

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ACCEPTED MANUSCRIPT

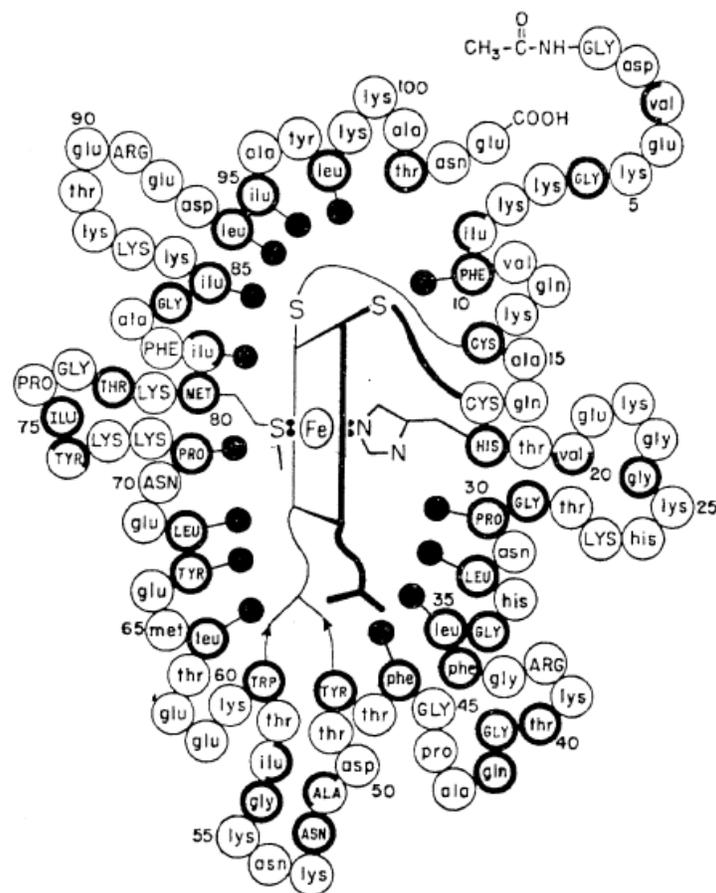


Figure 1

Figure 1. Heme packing diagram of the cytochrome c molecule. Heavy circles indicate side chains that are buried on the interior of the molecule, and attached black dots mark residues whose side chains pack against the heme, and dark half circles show groups that are half-buried at the surface. Arrows from try⁵⁹ and tyr⁴⁸ to the buried propionic acid group represent hydrogen bonds. From: Dickerson et al. [68], with permission.

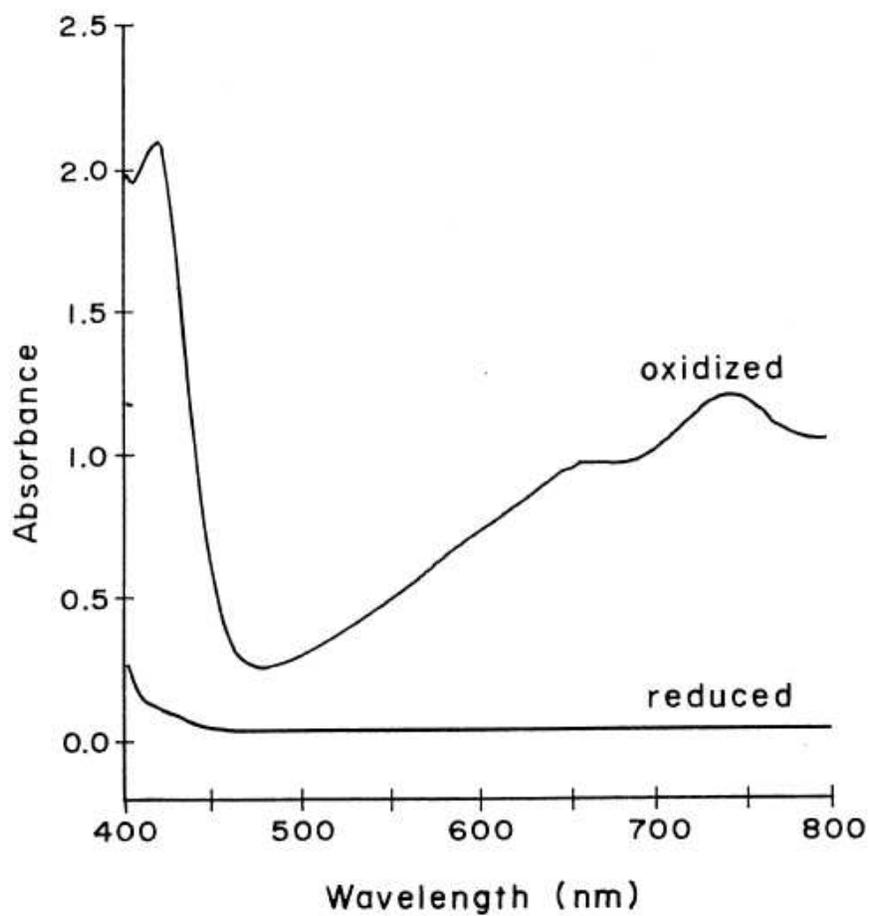


Figure 2

Figure 2. Absorption spectra of 0.05 mM oxidized and reduced ABTS in phosphate-citrate buffer, 50 mM each, at pH 5.4. Reduced ABTS is virtually colorless, whereas oxidized ABTS is dark green. From Everse et al. [24], with permission.

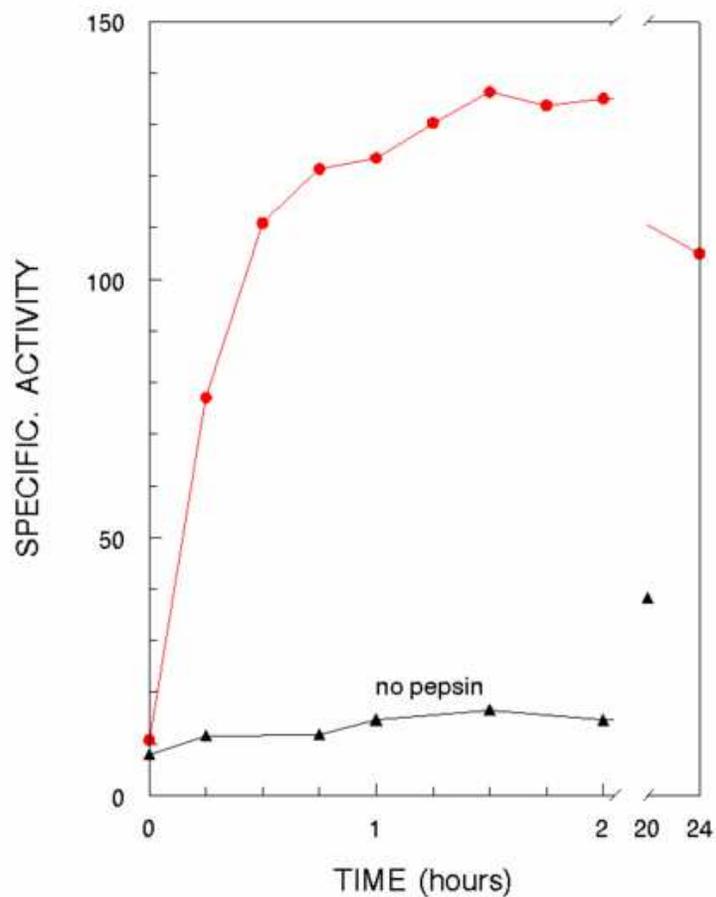


Figure 3

Figure 3. Progression of cytochrome c proteolysis with pepsin. Cytochrome c (0.1 mM) was treated with pepsin at room temperature and pH 2.6. At the indicated time intervals aliquots of the reaction mixture were removed and the peroxidase activity was determined as described in section 2.2.2.

Figure 4

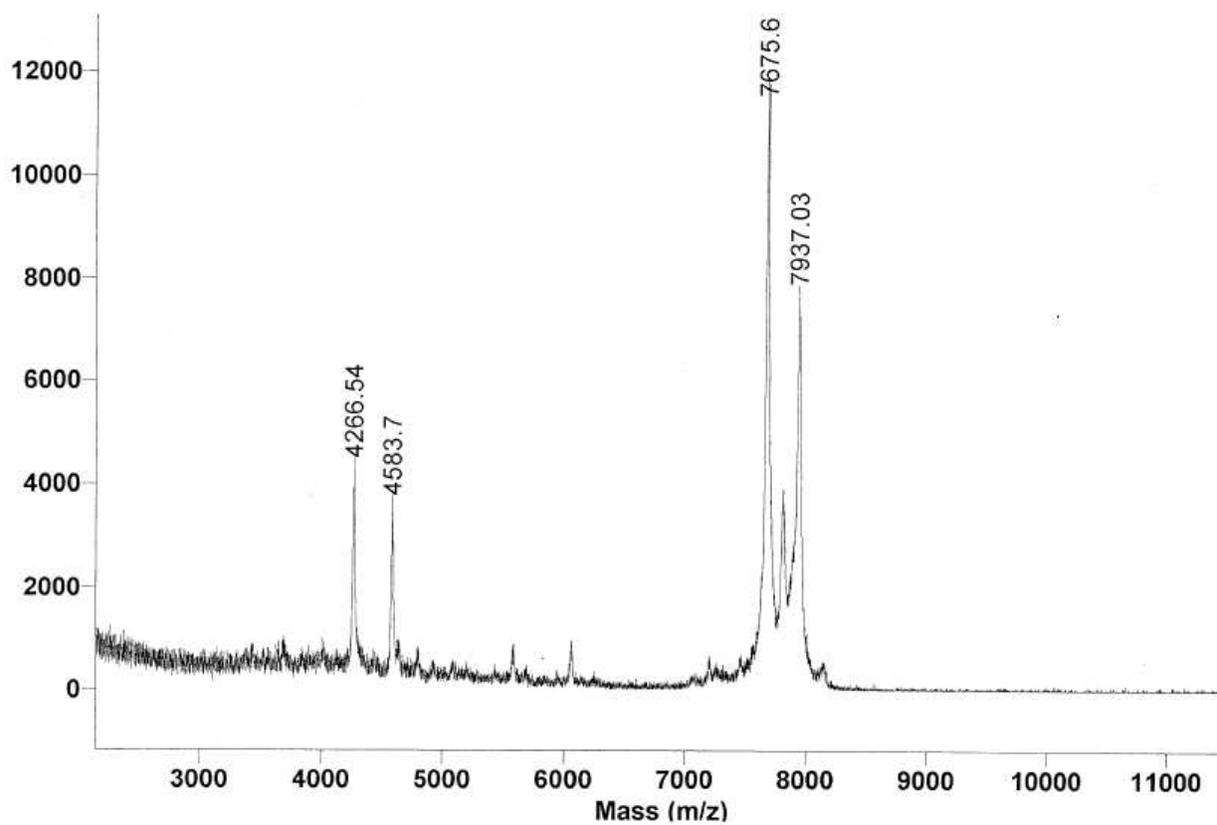


Figure 4. Mass spectroscopy results showing the products obtained from the proteolysis of cytochrome c with pepsin.

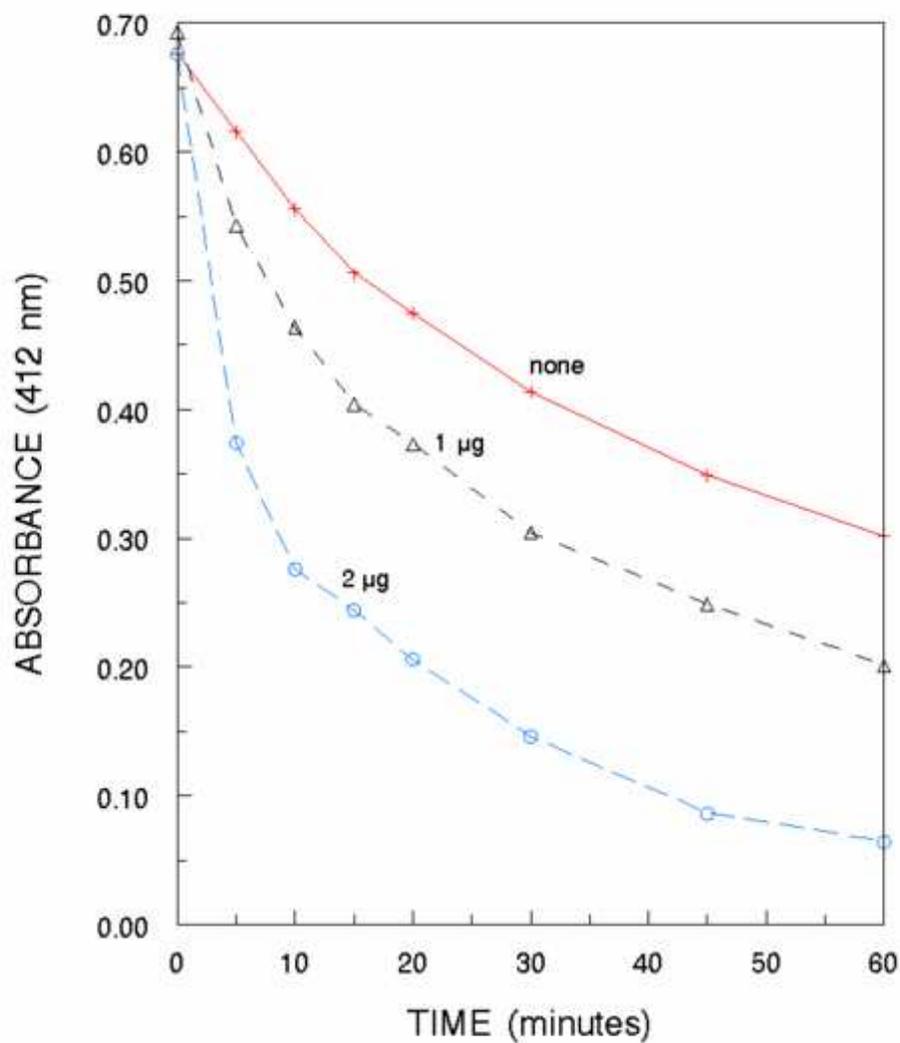


Figure 5

Figure 5. Oxidation of coenzyme A by 1 mM H_2O_2 and cytochrome c_{px} . Coenzyme A (30 :M in 50 mM tris-HCl buffer, pH 7.2, containing 1 mM DTPA) was incubated with or without the indicated amount of cytochrome c_{px} at 37EC for the indicated times. Remaining concentrations of coenzyme A were determined with the DTNB method as described in section 2.2.3.

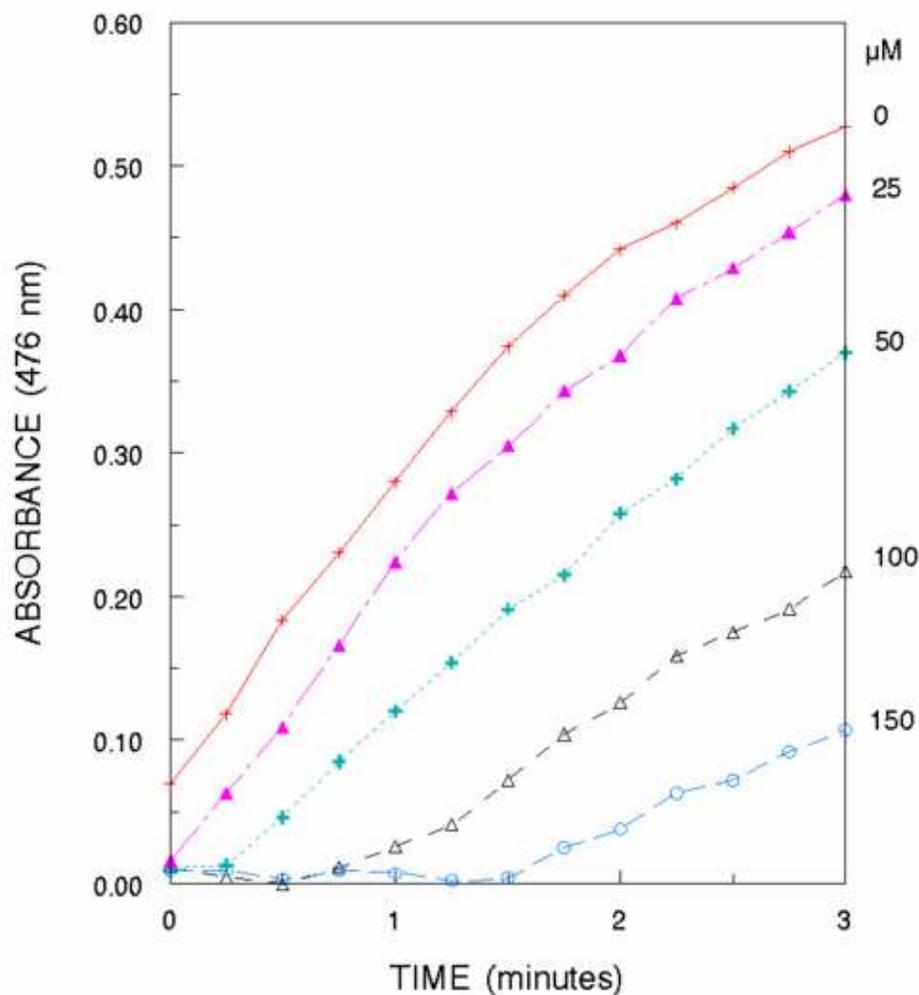


Figure 6

Figure 6. Inhibition of the cytochrome c_{px} -catalyzed oxidation of dopamine by cysteine.

Conditions: 800 μ M dopamine, 5 μ g cytochrome c_{px} , 5 mM H_2O_2 , and the indicated concentrations of cysteine in 1 ml 50 mM tris-HCl buffer, pH 7.2, containing 1 mM DTPA. Assays were done at 37°C.

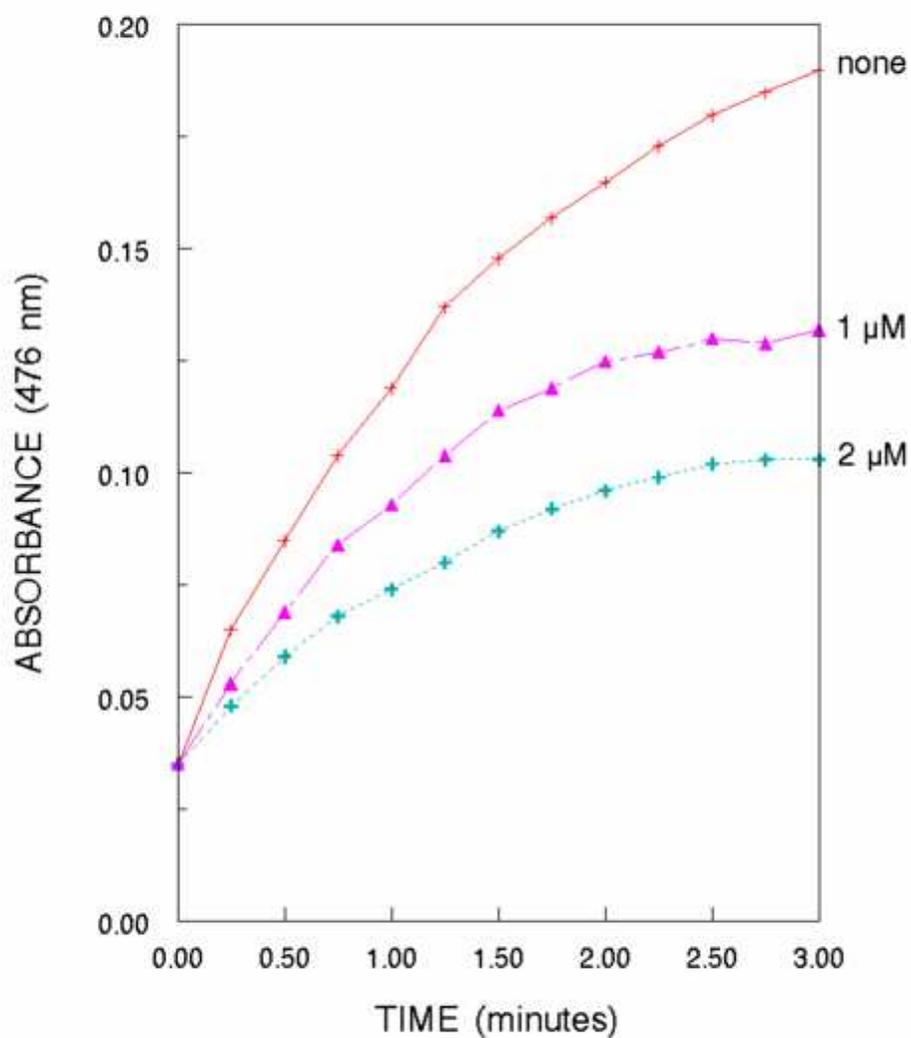


Figure 7

Figure 7. Inhibition of the cytochrome c_{px} -catalyzed oxidation of dopamine by curcumin.

Conditions were the same as in Figure 6, except the indicated concentrations of curcumin were used instead of cysteine.

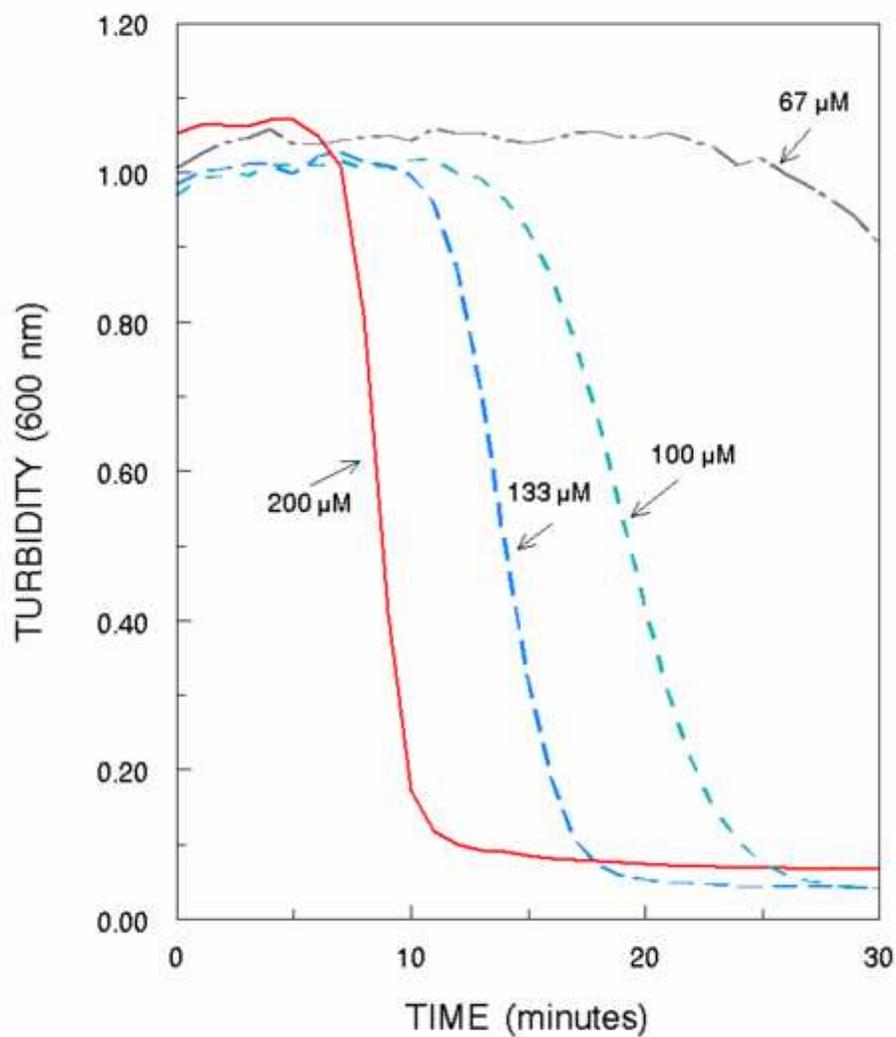


Figure 8

Figure 8. Hydrogen peroxide dependence of the cytolytic activity of cytochrome c_{px} . Cytolytic activity of cytochrome c_{px} toward rabbit erythrocytes was determined spectrophotometrically at 37EC as described in section 2.2.4.

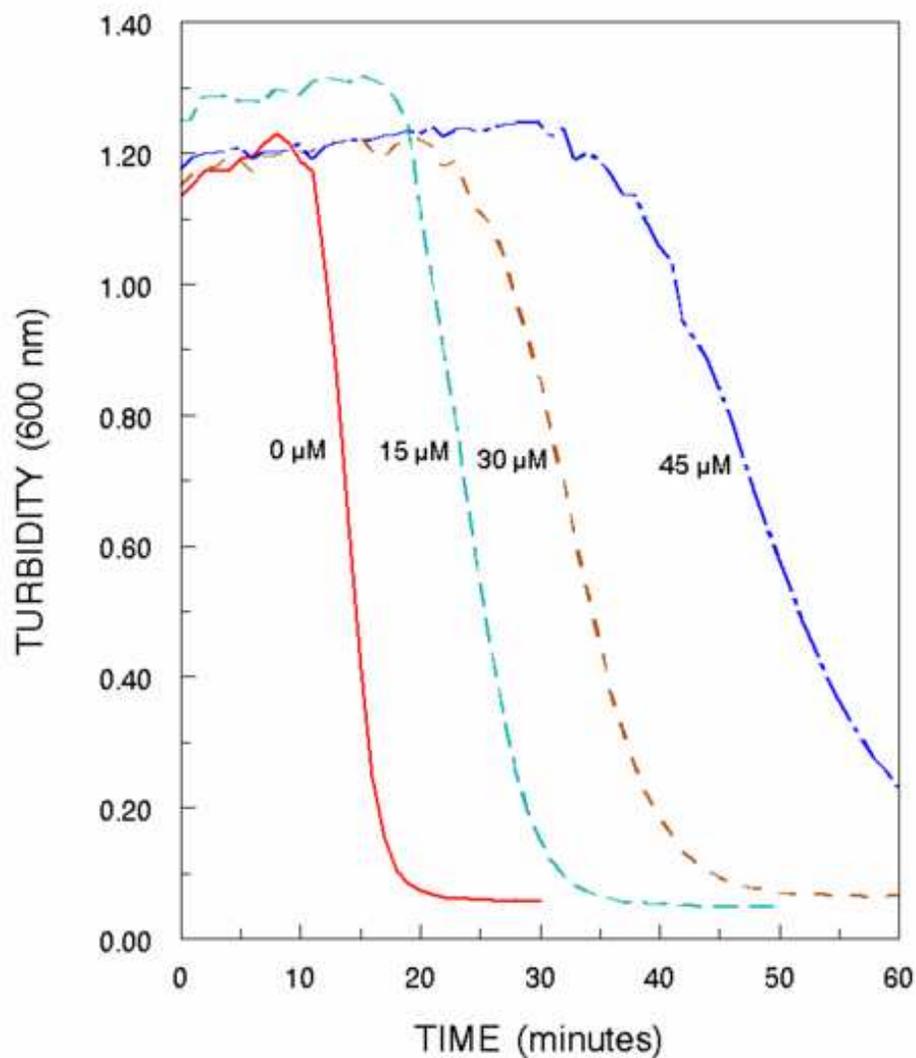


Figure 9

Figure 9. Inhibition of the cytolytic activity of cytochrome c_{px} by L-DOPA. Conditions: 2×10^6 rabbit erythrocytes, 0.2 mM H_2O_2 , 26 :M KI, 100 :g cytochrome c_{px} , and the indicated amounts of a 0.5 mM L-DOPA solution. Other conditions as described in section 2.2.4.