# Peroxidase Activity of Cytochrome *c*

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The peroxidase activity of cytochrome *c* was studied by using a chromogen, 2,2'-azinobis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS). Initial rate of ABTS oxidation formation was linear with respect to the concentration of cytochrome *c* between 2.5-10 $\mu$M and $H_2O_2$ between 0.1-0.5 mM. The optimal pH for the peroxidase activity of cytochrome *c* was 7.0-8.5. The peroxidase activity retained about 40% of the maximum activity when exposed at 60 °C for 10 min. The peroxidase activity showed a typical Michaelis-Menten kinetics for $H_2O_2$ which *Km* value was 29.6 mM. Radical scavengers inhibited the peroxidase activity of cytochrome *c*. The peroxidase activity was significantly inhibited by the low concentration of iron chelator, deferoxamine. The results suggested that the peroxidase activity was associated with iron in the heme of cytochrome *c*.

**Key Words :** Cytochrome *c*, Peroxidase, Radical scavenger, Iron chelator

## Introduction

Cytochrome *c* is one of the most well studied eukaryotic proteins.[1] The protein is synthesized in the cytoplasm. Post-translational addition of its heme moiety is catalyzed by heme lyase in the inner-membrane space of the mitochondrion.[2] Until recently, cytochrome *c* was believed to act solely as the penultimate electron-transfer protein of the eukaryotic respiratory chain. However, this protein has now been shown to play important roles in both apoptosis[3,4] and diseases associated with oxidative stress.[5]

Recent discoveries implicate cytochrome *c* in oxidative stress, which results from the run-away production of reactive oxygen species. The cellular damage induced by oxidative stress has been associated with several diseases, including Parkinson's disease (PD). Specially, it has been shown that cytochrome *c* is co-localized with $\alpha$-synuclein aggregates in Lewy bodies which are the pathological hallmarks of PD,[5] and that cytochrome *c* catalyzes the $H_2O_2$-induced aggregation of $\alpha$-synuclein.[5] Although all of these observation are archetypical results inducible by free radicals, it is not well understood how free radicals are generated by the effect of cytochrome *c* and what are the initial and cascading radical species responsible for these damage *in vivo*.

It has been reported that the reaction of hydrogen peroxide with heme proteins, such as cytochrome *c*, produces highly reactive ferryl-heme species that are capable of oxidizing biomolecules and initiating lipid peroxidation.[6,7] Recently, it was reported that hydrogen peroxide oxidized cytochrome *c* to a peroxidase compound I-type intermediate, in which one oxidizing equivalent is present as an oxoferryl heme species and the other as the protein tyrosyl radical.[8]

In this study the peroxidase activity of cytochrome *c* was investigated by using a chromogen, 2,2'-azinobis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS). We also studied the effects of pH, temperature, and the iron specific chelator on the peroxidase activity of cytochrome *c*.

## Materials and Methods

**Materials.** Bovine cytochrome *c*, deferoxamine (DFX) and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma. The diammonium salt of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) was obtained from Boehringer Mannheim. Chelex 100 resin (sodium form) was obtained from Bio-Rad. All materials were treated with Chelex 100 resin.

**Measurement of peroxidase activity of cytochrome *c*.** The peroxidase activity of cytochrome c was measured by using a chromogen, 2,2'-azinobis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS).[6] ABTS is water-soluble and has a strong absorption at 340 nm with a molar extinction coefficient $\varepsilon_{340}$ of $3.66 \times 10^4$ $M^{-1}cm^{-1}$.[9] On oxidation, ABTS forms a stable blue-green product presumed to be the cation radical. $ABTS^{+\cdot}$ is conveniently followed at $\lambda_{max}$ at 415 nm ($\varepsilon_{415}$= $3.6 \times 10^4$ $M^{-1}cm^{-1}$).[10] The assay mixture contained 10 mM potassium phosphate buffer (pH 7.4) and 50 $\mu$M ABTS and 0.1-0.5 mM hydrogen peroxide and 2.5-10 $\mu$M protein in a total volume of 1 mL. The reaction was initiated by addition of hydrogen peroxide and the increase in absorbance at 415 nm was measured by using a UV/Vis spectrophotometer (Shimazu 1601).

**Effects of radical scavengers and metal chelators on the peroxidase activity of cytochrome *c*.** 10 $\mu$M cytochrome *c* was allowed to react with 300 $\mu$M $H_2O_2$ in the presence of radical scavengers (azide, formate and ethanol) or iron chelators (EDTA and deferoxamine) at 37 °C. The peroxidase activity was determined by above described.

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## Results and Discussion

**Peroxidase activity of cytochrome *c*.** It has been shown previously that cytochrome *c* catalyzes the oxidation of various substrates such as ABTS and 4-aminoantipyrine by $H_2O_2$.[6]

Figure 1 shows the time-dependent change in optical absorption spectra obtained from a reaction mixture containing 50 $\mu$M ABTS, 0.3 mM $H_2O_2$ and 5 $\mu$M cytochrome c in 10 mM potassium phosphate buffer at pH 7.4. The reduction of ABTS absorbance is in agreement with the formation of $ABTS^{+\cdot}$ observed 415 nm. Initial rate of $ABTS^{+\cdot}$ formation monitored at 415 nm was linear with respect to the concentration of cytochrome *c* between 2.5-10 $\mu$M (Fig. 2) and $H_2O_2$ between 0.1-0.5 mM (Fig. 3). These data show that ABTS, a sulfonate anion, binds to cytochrome *c* with a relatively high affinity, apparently to an area near the active site where reactive oxygen species are produced.

**Effects of pH and temperature on the peroxidase activity.** The peroxidase activity was activated with the optimum pH range from 7.0 to 8.5 (Fig. 4). We examined the thermal stability for the peroxidase activity of the cyto-

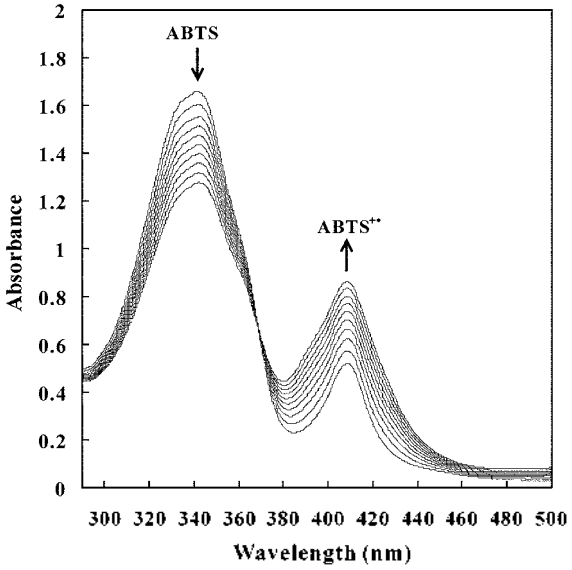


**Figure 1**. Electronic absorption spectra as a function of time. The spectra were obtained with a solution containing 50 $\mu$M ABTS and 300 $\mu$M $H_2O_2$ in 10 mM potassium phosphate buffer at pH 7.4. Each scan took 2 min. The arrows indicate the direction of absorbance change with time.

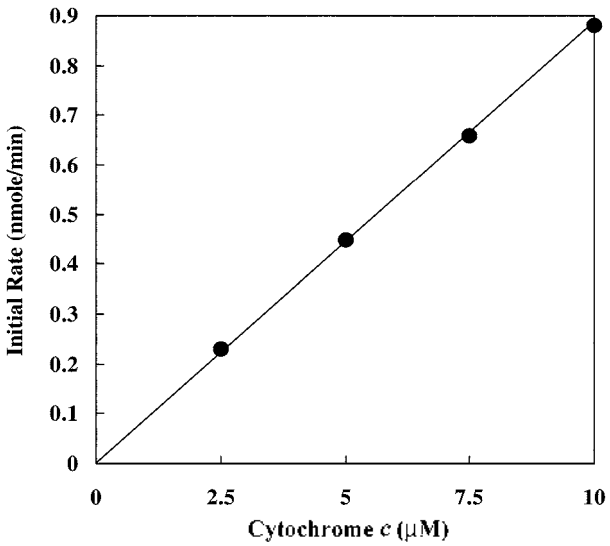


**Figure 2**. The effects of protein concentration on the rates of ABTS oxidation. The reaction mixture contained: 50 $\mu$M ABTS, 300 $\mu$M $H_2O_2$ and 2.5-10 $\mu$M cytochrome *c* in 10 mM potassium phosphate buffer at pH 7.4.

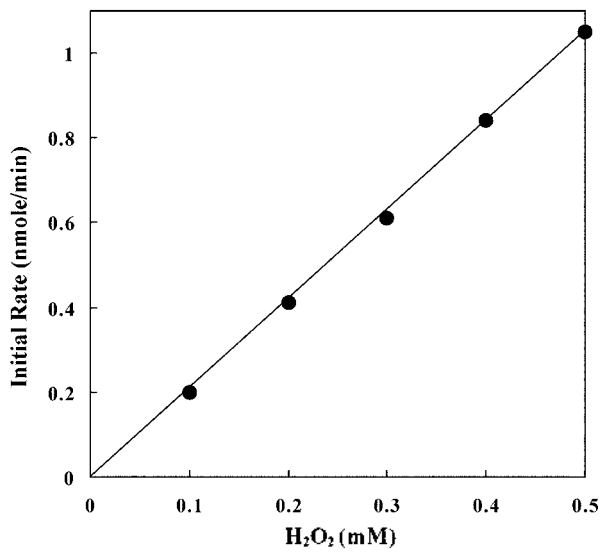


**Figure 3**. The effects of hydrogen peroxide concentration on the rates of ABTS oxidation. The reaction mixture contained: 50 $\mu$M ABTS, 0.1-0.5 mM $H_2O_2$ and 5 $\mu$M cytochrome *c* in 10 mM potassium phosphate buffer at pH 7.4.

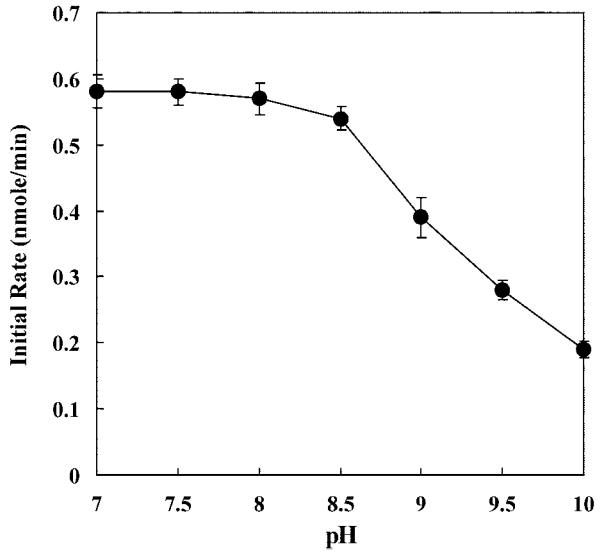


**Figure 4**. The effects of pH on the initial rates of ABTS oxidation of cytochrome *c*. The reaction mixtures contained 50 $\mu$M ABTS, 300 $\mu$M $H_2O_2$ and 5 $\mu$M cytochrome *c* in 10 mM potassium phosphate buffer (pH 7.0), or $NaHCO_3/CO_2$ (pH 7.6), or $Na_2CO_3$/$NaHCO_3$ (pH 8.0-10.0) buffer.

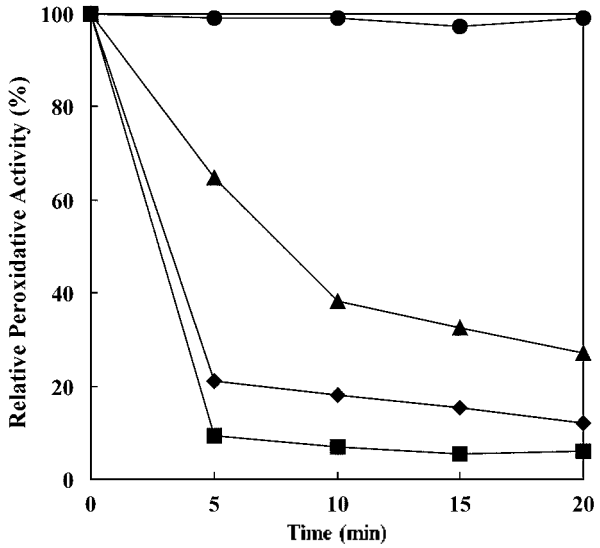


**Figure 5**. The thermal stability of cytochrome *c* for the peroxidase activity. Cytochrome *c* were placed in heating block at 40 °C (- ● -), 60 °C (- ▲ -), 80 °C (- ◆ -) and 100 °C (- ■ -) with indicated heating time. The reaction mixture contained 50 $\mu$M ABTS, 300 $\mu$M $H_2O_2$ and 5 $\mu$M cytochrome *c* in 10 mM potassium phosphate buffer at pH 7.4.

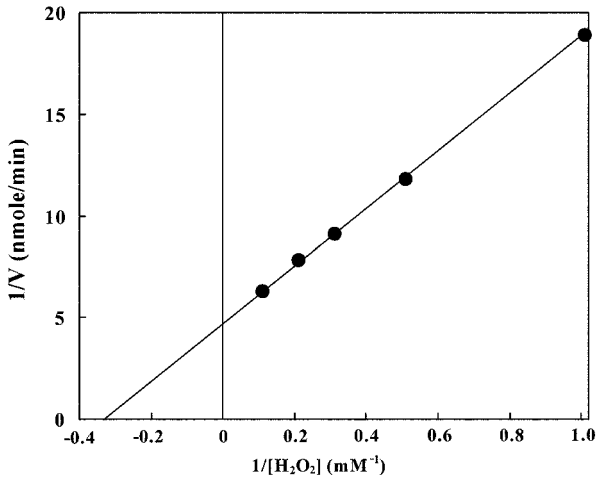


**Figure 6**. Double reciprocal plots of $H_2O_2$ concentrations *vs.* the peroxidase activity of the cytochrome *c*. Assays were run at 1 mM $H_2O_2$ in 10 mM potassium phosphate buffer at pH 7.4.

chrome *c*. The peroxidase activity retained about 40% of the maximum activity when exposed at 60 °C for 10 minutes (Fig. 5).

**Kinetic properties.** The peroxidase activity showed typical Michaelis-Menten kinetics for $H_2O_2$ which $K_m$ value was 29.6 mM (Fig. 6). It has been reported that the value of $K_m$ calculated for $H_2O_2$ in carboxymethylated cytochrome *c* at pH 7.0 is 25 mM.[12] The $H_2O_2$ *in vivo* is probably a direct product of $O_2^-$ dismutation and various oxidase reaction. It has been reported that the rate of $H_2O_2$ formation under physiological condition was 90 $\mu$M $H_2O_2$/min in liver at 22 °C.[13] Yim *et al.*[14] has been mention that at least 0.1 mM/min $H_2O_2$ will be produced continuously under physiological condition and at a much higher rate in adverse conditions

**Table 1**. Effect of radical scavengers on the formation of $ABTS^{+\cdot}$ by cytochrome *c* and hydrogen peroxide system

| Radical scavengers concentration | | $ABTS^{+\cdot}$ | |
|---|---|---|---|
| | | nmole/min | % |
| None | | 0.61 | 100 |
| Azide | 1 mM | 0.239 | 39 |
| | 5 mM | 0.172 | 28 |
| Formate | 1 mM | 0.566 | 93 |
| | 5 mM | 0.479 | 79 |
| Ethanol | 1 mM | 0.476 | 78 |
| | 5 mM | 0.326 | 53 |

The reaction mixture contained 10 mM potassium phosphate buffer (pH 7.4), 5 $\mu$M cytochrome *c*, 300 $\mu$M $H_2O_2$, 50 $\mu$M ABTS. The reaction was initiated by addition of $H_2O_2$ and incubated without or with radical scavengers at 37 °C.

such as hyperoxia or ischemia and reperfusion. Hence, the peroxidase activity of cytochrome *c* using $H_2O_2$ as a substrate will be operative *in vivo*.

**Effect of radical scavengers on the peroxidase activity.** The participation of radicals in the peroxidase activity of cytochrome *c* was studied by examining the protective effects of the radical scavengers such as azide, formate, and ethanol. When cytochrome *c* was incubated with $H_2O_2$ in the presence of various radical scavengers at 37 °C for 5 min, all scavengers inhibited the peroxidase activity of cytochrome *c* (Table 1). The result suggested that the radicals may be involved in this catalytic activity.

**Effects of metal chelators on the peroxidase activity.** Metal ions play a important role in the oxidative modification of proteins.[15] Effects of metal chelators on the peroxidase activity of cytochrome *c* was investigated. EDTA inhibited about 60% of the peroxidase activity at 1 mM while DFX at 10 $\mu$M inhibited 97% of the peroxidase activity. Deferoxamine (DFX) is an iron chelator used for removal of iron storage disease (thalasemias, iron poisoning, etc). DFX is also considered to be a potent free radical scavenger by preventing hydroxyl radical generation through the Fenton reation.[16] Low dose DFX has been improved survival in ischemia-reperfusion injury[17] and erythropoiesis in chronic hemodialysis patients.[18] However, large dose

**Table 2**. Effect of metal chelators on the formation of $ABTS^{+\cdot}$ by cytochrome *c* and hydrogen peroxide system

| Metal chelators concentration | | $ABTS^{+\cdot}$ | |
|---|---|---|---|
| | | nmole/min | % |
| None | | 0.61 | 100 |
| EDTA | 1 mM | 0.239 | 39 |
| | 5 mM | 0.022 | 4 |
| DFX | 0.01 mM | 0.065 | 11 |
| | 0.1 mM | 0.019 | 3 |

The reaction mixture contained 10 mM potassium phosphate buffer (pH 7.4), 5 $\mu$M cytochrome *c*, 300 $\mu$M $H_2O_2$, 50 $\mu$M ABTS. The reaction was initiated by addition of $H_2O_2$ and incubated without or with metal chelators at 37 °C.

treatment reduced survival and implied a toxic effect.[17] The peroxidase activity of cytochrome *c*, in part, may be responsible for the deleterious effects observed by mitochondria dysfunction. Therefore, we suggest that the low concentration of DFX may protect cells from the deleterious effects by the radicals generated in the presence of cytochrome *c* and hydrogen peroxide.

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## References

1. 1. Moore, G. R.; Pettigrew, G. W. *Cytochrome c: Evolution, Structure, and Physicochemical Aspects*; Springer-Verlag: Berlin, 1990.
2. Dumont, M. E.; Cardillo, T. S.; Hayes, M. K.; Sherman, F. *Mol. Cell Biol.* **1991**, *11*, 5487.
3. Liu, X.; Kim, C. N.; Yan, J.; Jemmerson, R.; Wang, X. *Cell* **1996**, *86*, 147.
4. Olteanu, A.; Patel, C. N.; Demon, M. M.; Kennedy, S.; Linhoff, M. W.; Minder, C. M.; Potts, P. R.; Deshmukh, M.; Pielak, G. J. *Biochem. Biophys. Res. Commun.* **2003**, *312*, 733.
5. Hashimoto, M.; Takeda, A.; Hsu, L. J.; Takenouchi, T.; Masliah, E. *J. Biol. Chem.* **1999**, *274*, 28849.
6. Radi, R.; Thomson, L.; Rubbo, H.; Prodanov, E. *Arch. Biochem. Biophys.* **1991**, *288*, 112.
7. Radi, R.; Sims, S.; Cassina, A.; Turrens, J. F. *Free Rad. Biol. Med.* **1993**, *15*, 653.
8. Lawrence, A.; Jones, C. M.; Wardman, P.; Burkitt, M. J. *J. Biol. Chem.* **2003**, *278*, 29410.
9. Rush, J. D.; Koppenol, W. H. *J. Am. Chem. Soc.* **1988**, *110*, 4957.
10. Childs, R. E.; Bardsley, W. G. *Biochem. J.* **1975**, *145*, 93.
11. Wolfenden, B. S.; Wilson, R. L. *J. Chem. Soc. Perkin Trans.* **1982**, *II*, 805.
12. Prasad, S.; Maiti, N. C.; Mazumdar, S.; Mitra, S. *Biochim. Biophys. Acta* **2002**, *1596*, 63.
13. Boveries, A.; Oshino, N.; Chance, B. *Biochem. J.* **1972**, *128*, 617.
14. Yim, M. B.; Chock, P. B.; Stadtman, E. R. *J. Biol. Chem.* **1993**, *268*, 4099.
15. Kang, J. H. *Bull. Korean Chem. Soc.* **2004**, *25*, 625.
16. Kamp, D. W.; Graceffa, P.; Pryor, W. A.; Weitzmn, S. A. *Free Radic. Biol. Med.* **1992**, *12*, 293.
17. DeBore, D. A.; Clark, R. E. *Ann. Thorac. Surg.* **1992**, *53*, 412.
18. Lee, C. T.; Liao, S. C.; Hsu, K. T.; Lam, K. K.; Chen, J. B. *Ren. Fail.* **1999**, *21*, 665.