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# PROTON STOICHIOMETRY OF THE CYTOCHROME *c* PEROXIDASE MECHANISM AS A FUNCTION OF pH

CURTIS W. CONROY and JAMES E. ERMAN
*Department of Chemistry, Northern Illinois University, DeKalb, Ill. 60115 (U.S.A.)*
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## Summary

The proton stoichiometry for the oxidation of cytochrome *c* peroxidase (ferrocytochrome *c*: hydrogen-peroxide oxidoreductase, EC 1.11.1.5) to cytochrome *c* peroxidase Compound I by $H_2O_2$, for the reduction of cytochrome *c* peroxidase Compound I to cytochrome *c* peroxidase Compound II by ferrocyanide, and for the reduction of cytochrome *c* peroxidase Compound II to the native enzyme by ferrocyanide has been determined as a function of pH between pH 4 and 8. The basic stoichiometry for the reaction is that no protons are required for the oxidation of the native enzyme to Compound I, while one proton is required for the reduction of Compound I to Compound II, and one proton is required for the reduction of Compound II to the native enzyme. Superimposed upon the basic stoichiometry is a contribution due to the perturbation of two ionizable groups in the enzyme by the redox reactions. The $pK_a$ values for the two groups are 4.9 ± 0.3 and 5.7 ± 0.2 in the native enzyme, 4.1 ± 0.4 and 7.8 ± 0.2 in Compound I, and 4.3 ± 0.4 and 6.7 ± 0.2 in Compound II.

## Introduction

The peroxidases, a group of heme-containing enzymes, catalyze the oxidation of a wide variety of compounds by $H_2O_2$ [1]. The catalytic mechanism involves two oxidized intermediates called Compound I and Compound II. $H_2O_2$ oxidizes the ferric form of the peroxidase to Compound I, which retains the two oxidizing equivalents of $H_2O_2$. Compound I is reduced to Compound II by various electron donors in a one electron step. A second one electron step reduces Compound II back to the native enzyme.

Yamada and Yamazaki [2] have measured the proton stoichiometry in the conversion of horseradish peroxidase (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) between the native enzyme and Compounds I and II.

They confirmed the observations of Schonbaum and Lo [3] that there is no proton change in the oxidation of horseradish peroxidase to Compound I by $H_2O_2$. In addition, the former authors showed that one proton is required during the conversion of Compound I to Compound II and a second proton is required during the reduction of Compound II back to the native enzyme.

While cytochrome *c* peroxidase (ferrocytochrome *c*: hydrogen-peroxide oxidoreductase, EC 1.11.1.5) follows the same basic mechanism involving two oxidized intermediates, the spectral properties of the intermediates are quite distinct from those of horseradish peroxidase [4—6]. In this paper, we will refer to the two oxidized intermediates of cytochrome *c* peroxidase as cytochrome *c* peroxidase Compound I and cytochrome *c* peroxidase Compound II. Compound I is the first intermediate observed after reaction of the native enzyme with hydroperoxides [6,7] and is distinguished from the initial compounds of other hydroperoxidases by the fact that its Soret absorbtion band is more intense than that of the native enzyme (solutions of Compound I are red rather than the green found with other hydroperoxidase Compounds I) [4] and by a 'free radical-like' EPR spectrum [5]. Compound II is formed by the one electron reduction of Compound I [6]. Compound II appears to be an equilibrium mixture of two species, one in which the oxidizing equivalent resides on the heme iron atom and is predominant in neutral and basic solution while the second species retains the oxidizing equivalent as an organic free radical, which is more stable in acid solution [6]. The equilibrium between these two species is established rapidly. In this paper we will treat Compound II as a single enzyme intermediate whose properties vary as a function of pH.

To determine whether the differences between cytochrome *c* peroxidase and other hydroperoxidases extend to the proton stoichiometry of the oxidation-reduction steps, we have investigated the proton balance for the individual steps in the cytochrome *c* peroxidase mechanism.

## Materials and Methods

Cytochrome *c* peroxidase was isolated from commercial baker's yeast and crystallized by dialyzing against distilled, deionized water according to the method of Yonetani and coworkers [8,9]. Solutions of enzyme were prepared in 0.1 M $KNO_3$ and the concentration determined spectrophotometrically using an extinction coefficient of 95 $mM^{-1} \cdot cm^{-1}$ at 408 nm [10]. Potassium ferrocyanide solutions were prepared by weight using reagent grade material. All solutions were prepared with deionized, distilled water.

The pH dependence of the cytochrome *c* peroxidase spectrum was determined with a sample cuvette which had ports for pH electrodes and a micrometer syringe. The pH of the sample solution could be adjusted and measured while remaining in the spectrophotometer. A solution of enzyme was adjusted to the desired initial pH and identical samples were placed in both a reference cuvette and the sample cuvette. After obtaining a base line, the pH in the sample cuvette was changed and the difference spectrum recorded. Difference spectra were obtained at about 0.2 pH intervals between pH 4 and 8. In any single experiment, the volume of acid or base to adjust the pH was less than 0.5% of the total solution volume.

The proton uptake experiment were carried out in a closed vessel with $N_2$ flowing over the surface of the reactant solution. The container has ports for a glass electrode and a calomel reference electrode. The pH of the solution was measured with a Radiometer model 26 pH meter. There were additional ports fitted with micrometer syringes for the addition of reactants and standardized HCl or KOH for pH adjustment. The solution was stirred with a magnetic stirrer.

To determine the proton stoichiometry in the reaction between cytochrome *c* peroxidase and $H_2O_2$ to form Compound I, a known amount of $H_2O_2$ was added to an excess of cytochrome *c* peroxidase while monitoring the pH of the reaction medium. If a pH change occurred, the solution was titrated back to the original pH with either standard acid or base and the number of protons taken up or liberated in the reaction per mol of enzyme reacted was calculated, $n_1$. The value of $n_1$ is defined as positive for consumption of protons and negative for proton liberation during the reaction. An average of four determinations were carried out at each condition investigated. The standard deviation for $n_1$ averaged ±0.11.

The proton consumption per mol of Compound I reduced to Compound II, $n_2$, and per mol of Compound II reduced to the native enzyme, $n_3$, are more difficult to determine because Compound I and Compound II are reduced at comparable rates by all reducing agents investigated [11]. As a consequence $n_2$ and $n_3$ cannot be determined independently. In order to simultaneously determine $n_2$ and $n_3$, Compound I was formed by adding a known quantity of $H_2O_2$ to excess cytochrome *c* peroxidase. The pH of the resulting solution was rapidly adjusted to the desired pH. A known aliquot of ferrocyanide, sufficient to reduce about 20—25% of the oxidizing equivalents of Compound I, was added and the amount of acid required to maintain the pH constant was noted. Additional aliquots of ferrocyanide were added stepwise, adjusting the pH after each ferrocyanide addition until the enzyme was completely reduced to the ferric state. The amount of Compound I reduced to Compound II and the amount of Compound II reduced to ferric enzyme after each addition of ferrocyanide was calculated from the amount of ferrocyanide added and the known rate of reduction of each intermediate [11].

For example, in one experiment at pH 4.5, beginning with 3 ml of 91 $\mu$M Compound I (273 nmol), five aliquots of 100 nmol and one aliquot of 50 nmol ferrocyanide were added to the Compound I solution. The total of 550 nmol ferrocyanide are sufficient to reduce the Compound I to the native enzyme via Compound II. Under these experimental conditions, the rates of reduction of Compound I to Compound II and Compound II to the native enzyme are $(3.2 \pm 0.7) \cdot 10^6$ and $(1.1 \pm 0.3) \cdot 10^6\ M^{-1} \cdot s^{-1}$, respectively [11]. Using these rate constants and the concentration of reactants, the concentration of Compound I, Compound II, and the native enzyme at the completion of the reaction following the addition of each aliquot of ferrocyanide can be calculated. In this example the amount of Compound I is 183, 104, 46, 14, 0, and 0 nmol while that of Compound II is 85, 139, 156, 123, 46, and 0 nmol after each of the six ferrocyanide additions. The amount of native enzyme can be calculated from the conservation of mass. The proton consumption was measured by back titrating to pH 4.5 with standard acid after completion of the reaction fol-

lowing addition of each aliquot of ferrocyanide. The cumulative amount of protons consumed in this experiment were 179, 331, 491, 631, 706, and 706 nmol after each of the six ferrocyanide additions. Values of $n_2$ and $n_3$ can be determined from this data by noting that the total proton consumption is equal to $n_2$ times the amount of Compound I reduced to Compound II plus $n_3$ times the amount of Compound II reduced to native enzyme, Eqn. 1,

$$[H^+]_{Total} = n_2([\text{Compound I}]_0 - [\text{Compound I}]) + n_3[\text{cytochrome } c \text{ peroxidase}] \quad (1)$$

where $[H^+]_{Total}$ is the total concentration of protons consumed after each step in the reduction, $[\text{Compound I}]_0$ is the initial concentration of Compound I, while [Compound I] and [cytochrome *c* peroxidase] are the concentration of Compound I and native enzyme remaining after reduction by each aliquot of ferrocyanide. Dividing Eqn. 1 by ($[\text{Compound I}]_0 - [\text{Compound I}]$), values of $n_2$ and $n_3$ can be obtained from the intercept and a slope of a plot of $[H^+]_{Total}/([\text{Compound I}]_0 - [\text{Compound I}])$ versus [cytochrome *c* peroxidase]/($[\text{Compound I}]_0 - [\text{Compound I}]$). An average of four experiments were performed at each condition investigated. The average standard deviation for $n_2$ and $n_3$ is ±0.12.

## Results and Discussion

The net reaction for the oxidation of ferrocyanide by $H_2O_2$ is given by Eqn. 2.

$$2H^+ + H_2O_2 + 2Fe(CN)_6^{4-} \rightarrow 2H_2O + 2Fe(CN)_6^{3-} \quad (2)$$

The proton stoichiometry of the reaction was confirmed by carrying out the reaction in the presence of catalytic amounts of cytochrome *c* peroxidase (CcP). Between pH 5 and 8, the reaction requires two protons as shown in Eqn. 2 but the requirement decreases to 1.75 protons at pH 4 due to the partial protonation of ferrocyanide [12].

The overall reaction involves a three-step mechanism shown in Eqns. 3—5.

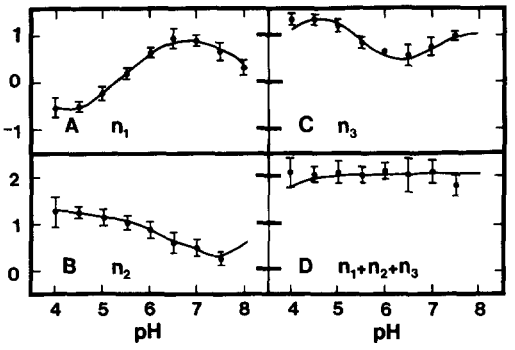
$$n_1H^+ + H_2O_2 + \text{CcP} \rightarrow \text{Compound I} \quad (3)$$

$$n_2H^+ + \text{Compound I} + Fe(CN)_6^{4-} \rightarrow \text{Compound II} + Fe(CN)_6^{3-} \quad (4)$$

$$n_3H^+ + \text{Compound II} + Fe(CN)_6^{4-} \rightarrow \text{CcP} + Fe(CN)_6^{3-} \quad (5)$$

Values of $n_1$, $n_2$, and $n_3$ as a function of pH are shown in Figs. 1A—1C. The sum of $n_1$, $n_2$, and $n_3$ is shown in Fig. 1D. Within experimental error, the sum agrees with the theoretical proton requirement for the net reaction, Eqn. 2, which is indicated by the solid line in Fig. 1D.

The results for cytochrome *c* peroxidase appear to be quite distinct from those obtained for horseradish peroxidase [2,3]. For horseradish peroxidase, $n_1$ equals zero between pH 5.3 and 8.3 while $n_2$ and $n_3$ both have values near 1 at pH 5.50 and 6.85, the only two pH conditions investigated [2]. However, the differences obtained between cytochrome *c* peroxidase and horseradish peroxidase can be simply explained by postulating the existence of two ionizable groups in cytochrome *c* peroxidase which have their $pK_a$ values



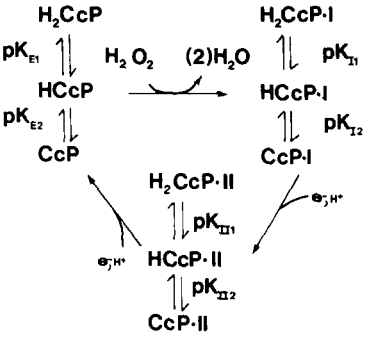




Fig. 1. (A) Proton stoichiometry of the reaction between cytochrome *c* peroxidase and $H_2O_2$, $n_1$ is defined by Eqn. 3 in the text. (B) Proton stoichiometry for the conversion of Compound I to Compound II; $n_2$ is defined by Eqn. 4 in the text. (C) Proton stoichiometry for the conversion of Compound II to cytochrome *c* peroxidase; $n_3$ is defined by Eqn. 5 in the text. (D) The sum of $n_1$, $n_2$, and $n_3$ which equals the proton stoichiometry for the oxidation of ferrocyanide by $H_2O_2$, Eqn. 2 in the text.

Fig. 2. A mechanism which accounts for the pH dependence of the proton stoichiometry for the conversion of cytochrome *c* peroxidase (CcP) to Compound I (CcP · I), Compound I to Compound II (CcP · II) and Compound II back to the native enzyme. Two ionizable groups in each species are perturbed during the redox reactions.

perturbed during the conversion from one oxidized form of the enzyme to another. Either the two groups do not exist in horseradish peroxidase or their $pK_a$ values remain the same in the various oxidized states. The scheme for cytochrome *c* peroxidase is shown in Fig. 2. Values of the acid dissociation constants defined in Fig. 2 were obtained by fitting the proton uptake data to equations of the form shown in Eqn. 6 for $n_1$.

$$n_1 = \frac{2 + K_{I1}/[H^+]}{1 + K_{II}/[H^+] + K_{I1}K_{I2}/[H^+]^2} - \frac{2 + K_{E1}/[H^+]}{1 + K_{E1}/[H^+] + K_{E1}K_{E2}/[H^+]^2} \qquad (6)$$

The equations for $n_2$ and $n_3$ also take into account the proton required for the unperturbed reaction and for the protonation of ferrocyanide below pH 5 [12]. The $pK_a$ values for the native enzymes, $pK_{E1}$ and $pK_{E2}$, are 4.9 ± 0.3 and 5.7 ± 0.2, respectively. For Compound I, $pK_{I1}$ equals 4.1 ± 0.4 while $pK_{I2}$ equals 7.8 ± 0.2. For Compound II, $pK_{II1}$ and $pK_{II2}$ are 4.3 ± 0.4 and 6.7 ± 0.2, respectively. The solid lines in Fig. 1 were calculated using the above $pK_a$ values.

It has previously been noted that the spectrum of cytochrome *c* peroxidase shows small variations over the pH range 4—8 [13,14]. The previous studies were not precise enough to obtain good titration curves. In order to determine whether there is a correlation between the p*K* values determined from the proton uptake data and the spectral changes in cytochrome *c* peroxidase we have reinvestigated the pH dependence of the enzyme spectrum. The difference spectra between the enzyme at various pH values between pH 4.80 and 6.80 and the enzyme at pH 6.02 are shown in Fig. 3. Between pH 4.8 and 6.0, good isosbestic points are obtained at 398 and 422 nm. Below pH 4.8, the isosbestic points shift, and the intensity of the Soret maximum decreases slightly. This is probably due to the beginning of the changes which cause the rapid denaturation of the enzyme below pH 4. Between pH 6.0 and 7, the isosbestic point at

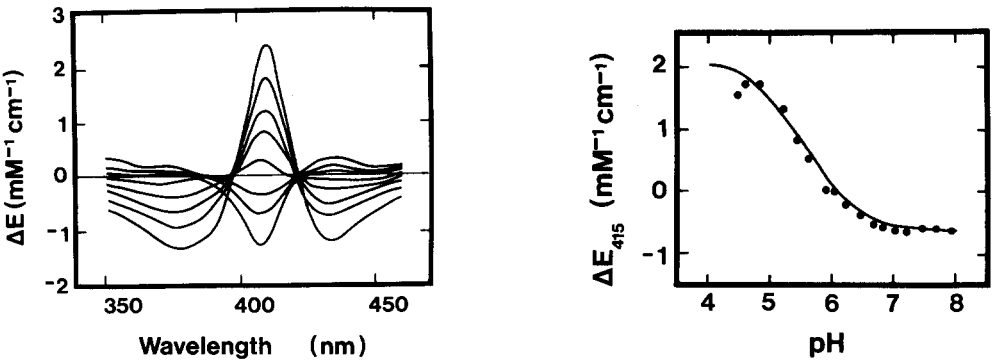



Fig. 3. Difference spectra between cytochrome *c* peroxidase at various pH values and cytochrome *c* peroxidase at pH 6.02. The pH values starting with the spectrum with the most positive extinction coefficient at 410 nm and proceeding in order of decreasing extinction coefficients at 410 nm are 4.80, 5.18, 5.41, 5.62, 5.88, 6.25, 6.47, and 6.80.

Fig. 4. The pH dependence of the difference extinction coefficient at 415 nm. Values were obtained relative to the value at pH 6.02. The data fit a single ionization with an apparent $pK_a$ of 5.5 ± 0.1.

398 nm shifts fo 386 nm and between pH 7 and 8 (not shown), three new isosbestic points are established at 351, 415, and 461 nm. The spectral changes above pH 7 indicate the beginnings of complex transitions which cause substantial perturbations of the spectrum above pH 8 and include denaturation of the enzyme (Dhaliwal, B. and Erman, J., unpublished data). The difference in extinction coefficient at 415 nm of cytochrome *c* peroxidase at various pH values and the enzyme at pH 6 is shown in Fig. 4. The spectral data were fit to a single ionization curve with an apparent p*K* value of 5.5 ± 0.1 in good agreement with the value for $pK_{E2}$ found from the proton uptake data.

Compound I decays slowly with time [15] and the pH dependence of its spectrum could not be determined with as great a precision as that for the native enzyme. Within the accuracy of the measurements, ±2 $mM^{-1} \cdot cm^{-1}$, the spectrum of Compound I is independent of pH between 4 and 8.

There is evidence to suggest that the oxidize site in cytochrome *c* peroxidase Compound II equilibrates between the heme iron and an organic residue in the protein [6]. At pH 8, the calculated absorption spectrum of Compound II resembles that of Compound I. As the pH is lowered, the spectrum begins to be converted to that of the native ferric enzyme with the appearance of a 'free radical-like' EPR spectrum [6]. The pH dependence of the transition has not been reported in detail. The spectrum of Compound II cannot be measured directly, but must be calculated from the changes in absorbance during the reduction of Compound I to the native enzyme via Compound II. The calculated values of the extinction coefficient of Compound II at 424 nm as a function of pH are shown in Fig. 5. These data were obtained from the reduction of Compound I by ferrocyanide [11]. The precision of the calculations are limited by the accuracy of the relative reduction rates for Compound I and Compound II. The extinction coefficient of Compound II was only determined within ±5 $mM^{-1} \cdot cm^{-1}$. The pH dependence of the extinction coefficient is probably due to the ionization of a specific group in the protein. The investigation could not be extended to low enough pH values to determine the low pH

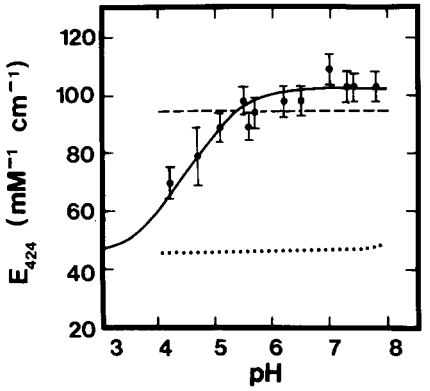



**Fig. 5. The pH dependence of the extinction coefficient of Compound II at 424 nm. The dotted line is the extinction coefficient of the native enzyme and the dashed line is the extinction coefficient of Compound I at 424 nm as a function of pH.**

limit for the extinction but it must lie between 0 and 65 $mM^{-1} \cdot cm^{-1}$, restricting the apparent $pK_a$ to the range 3.8—4.8. If it is assumed that the spectrum of the acidic form of Compound II is similar to that of the native ferric enzyme [6], then the $pK_a$ value is equal to 4.5, in good agreement with $pK_{II1}$ determined from the proton uptake data.

The mechanism postulated to account for the pH dependence of the proton stoichiometry for the oxidation and reduction of cytochrome *c* peroxidase is consistent with the kinetic properties of the enzyme. An ionizable group with a $pK_a$ of 5.5 ± 0.1 strongly influences the rate of reaction between the enzyme and $H_2O_2$ [7] fluoride [14,16] and cyanide [17]. A second ionizable group with $pK_a$ value of 5.1 ± 0.2 was found necessary to account for microscopic reversibility and proton stoichiometry in the fluoride-cytochrome *c* peroxidase reaction [16] although it had no detectable effect on the rate of association.

The rate of oxidation of dicyano-bis(1,10-phenanthroline) iron (II) by the oxidized intermediates of cytochrome *c* peroxidase is most rapid when ionizable group in the enzyme is protonated [18]. The apparent $pK_a$ of the group is 4.5 ± 0.2 in Compound II [18], in good agreement with $pK_{II1}$ determined in this study. In Compound I, the $pK_a$ of the kinetically important group was 4.8 ± 0.2 [18] a value somewhat higher than $pK_{I1}$ determined in this study. However, kinetic studies on Compound I are difficult [11,18] and it is probable that the same group influences both the kinetics and the proton stoichiometry. The groups denoted by $pK_{I2}$ and $pK_{II2}$ in Fig. 2 do not influence the kinetic properties of the oxidized intermediates.

Although we have obtained evidence for two ionizable groups and have measured two apparent acid dissociation constants in each of the three redox states of the enzyme, we cannot assign particular $pK_a$ values to the individual groups in each redox state. In fact, we are not even sure if we are monitoring the same two groups in each of the redox states. Oxidation of compounds can alter $pK_a$ values by many orders of magnitude. For example, the free radical in Compound I and Compound II is thought to be due to the oxidation of a tyrosine or tryptophan side chain [5,6,19,20]. If tyrosine is oxidized to a phenoxy radical, the $pK_a$ of the phenol groups would change from about 10 to less than —1 in

the radical state [21]. If a tryptophan radical were produced, the $pK_a$ of the indole ring would change from greater than 14 to about 4 in the oxidized radical [22]. The $pK_a$ value of 4 for the tryptophanyl radical is especially intriguing because of the occurrence of the groups with $pK_a$ 4.5 in Compound I and Compound II.

We want to emphasize that we do not have any direct evidence for associating the various $pK_a$ values with any particular groups in the enzyme, but we can speculate about the assignments. Upon oxidation of the heme iron, one of the two groups observed in the native enzyme may have its $pK_a$ value shifted outside the range of investigation. This would most likely be the group with $pK_a$ 5.5 since it seems to interact with the heme more strongly than the other group, altering both the absorption spectrum and the kinetic properties of the native enzyme. Upon formation of Compound I, a tryptophanyl radical could be formed, producing the group with $pK_a$ 4.5. This group is also present in Compound II. The $pK_a$ values of 5.0, 7.8, and 6.7 in cytochrome *c* peroxidase, Compound I, and Compound II, respectively, would be associated with a single group whose proton affinity is dependent upon the redox state and/or conformational state of the enzyme. This is a logical assignment since these three groups have similar properties in that they are only apparent in the proton stoichiometry for the conversion of one redox state of the enzyme to another. They do not influence the kinetic or spectral properties of the enzyme in any of its three redox states. We do not think that these $pK_a$ values are associated with the residue which forms the radical since the shifts in $pK_a$ are in the wrong direction in proceeding from the most reduced state to the oxidized state and not nearly as large as those observed in the model compound studies [21,22].

Of course, other assignments are possible. If a tyrosyl radical is produced rather than tryptophanyl radical, neither the original tyrosine or the tyrosyl radical would ionize in the pH range of this investigation. In this case we would associate the $pK_a$ values of 5.5, 4.5, and 4.5 to one group and the values of 5.0, 7.8, and 6.7 to the second group in cytochrome *c* peroxidase, Compound I, and Compound II, respectively. The reason primarily being that the first group of $pK_a$ values strongly influences the properties of the heme while the second group does not.

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