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OXIDATION OF DICYANO-BIS(1,10 PHENANTHROLINE) IRON(II) BY COMPOUNDS I AND II OF CYTOCHROME *c* PEROXIDASE

JAMES E. ERMAN

Department of Chemistry, Northern Illinois University, DeKalb, Illinois 60115 (U.S.A.)

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Summary

The apparent bimolecular rate constant for the oxidation of dicyano-bis(1,10 phenanthroline) iron(II) by compound II of cytochrome *c* peroxidase (ferrocytochrome *c*; hydrogen-peroxide oxidoreductase EC 1.11.1.5) has been measured over the pH range 2.5—11.0 at 0.1 M ionic strength, 25°C, by the stopped-flow technique. An ionizable group in the enzyme, with a pK_a of 4.5, strongly influences the electron transfer rate between the ferrous complex and the oxidized site in the enzyme. The electron transfer is fastest when the group is protonated, with a rate constant of $2.9 \cdot 10^5$ $M^{-1} \cdot s^{-1}$. The rate constant decreases over three orders of magnitude when the proton dissociates.

The apparent bimolecular rate constant for the oxidation of the ferrous complex by compound I of cytochrome *c* peroxidase was determined between pH 3.5 and 6. Under all conditions where this rate constant could be measured it was about three times larger than that for the oxidation by compound II.

In order to elucidate the mechanism of electron transfer reactions mediated by cytochrome *c* peroxidase (ferrocytochrome *c*: hydrogen-peroxide oxidoreductase, EC 1.11.1.5), we have previously investigated the oxidation of ferrocyanide by the oxidized intermediates of the enzyme [1]. One of the primary goals of that investigation was to determine the pH dependence of the electron transfer rates so as to establish whether acidic or basic groups in the enzyme could influence the reactions. However, the interpretation of the pH dependence of the rate constants was not definitive due to strong electrostatic interactions between the enzyme and the highly charged ferrocyanide ion. In fact, using extended Debye-Huckel expressions for the ionic strength dependence of the rate constants and a simple conducting-sphere model to determine the electrostatic free energy of activation at zero ionic strength, the pH dependence of the rate constants could be explained on the basis of electrostatic

interactions alone, without invoking the involvement of acidic or basic groups in the enzyme.

In view of the uncertainties involved in the application of the above model, it is of importance to experimentally determine the pH dependence of the electron transfer rates to the enzyme, under conditions which minimize electrostatic interactions. This report presents the pH dependence of the oxidation of dicyano-bis(1,10 phenanthroline) iron(II) by compounds I and II of cytochrome *c* peroxidase*. Dicyano-bis(1,10 phenanthroline) iron(II) has zero net charge and the rate constants are not affected by primary kinetic salt effects.

Materials and Methods

Preparation of enzyme, hydrogen peroxide, and buffer solutions was the same as previously described [1]. Enzyme concentrations for the kinetic studies were usually near 0.5 μM.

Dicyano-bis(1,10 phenanthroline) iron(II) dihydrate was purchased from G.F. Smith Chemical Company and used without further purification. The uncharged ferrous complex is relatively insoluble in water. An aqueous solution of the complex, prepared by stirring an excess of the solid complex in distilled water for a period of 24 h at room temperature, had a concentration of about 40 μM. A methanol solution, prepared in the same manner, had a concentration of 3.6 mM. For the kinetic studies, a 2 mM solution of the complex in methanol was prepared by weight and small aliquots of the methanol solution were added to the aqueous buffers just prior to introduction into the stopped-flow apparatus. Final concentrations of the ferrous complex ranged from 10 to 200 μM and the methanol concentrations ranged from 0.5 to 10%. At the higher concentrations of the ferrous complex, a slow precipitation was noticed over the period of an hour. This did not interfere in the kinetic analysis except between pH 7.5 and 8.5, where the oxidation-reduction reactions are the slowest.

Kinetic measurements were performed on a Durrum-Gibson stopped-flow spectrophotometer. The reaction was observed either at 414.5 or 424 nm. To increase the pH range beyond the region where the enzyme is stable, the unbuffered compound I was placed in one drive syringe. Buffered solutions of the ferrous complex were placed in the second drive syringe. The pH of the solutions was measured after mixing the two reactants. Compound I was formed just prior to introduction into the stopped-flow apparatus by adding slightly less than a stoichiometric amount of hydrogen peroxide to cytochrome *c* peroxidase.

All reactions were carried out at 25.0 ± 0.1 °C and 0.10 ± 0.01 M ionic strength.

Analysis of the data was facilitated by the use of a non-linear least-squares curve fitting program, KINET, obtained from Dr J.L. Dye of Michigan State University [2].

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* The term "compound I" refers to the primary intermediate oxidized two equivalents above the native ferric form of cytochrome *c* peroxidase. This intermediate has been called "compound ES" [3] or "complex ES" [7] by Yonetani and coworkers. "Compound II" refers to the secondary intermediate formed in the one electron reduction of compound I.

Results

Cytochrome *c* peroxidase follows a three step mechanism similar to the classical peroxidase mechanism shown in Scheme I [3].

SCHEME I

$$CcP + H_2O_2 \xrightarrow{k_1^{app}} CcP\text{-}I$$

$$CcP\text{-}I + Fe^{2+} \xrightarrow{k_2^{app}} CcP\text{-}II + Fe^{3+}$$

$$CcP\text{-}II + Fe^{2+} \xrightarrow{k_3^{app}} CcP + Fe^{3+}$$

CcP represents the native enzyme. CcP-I represents compound I of cytochrome *c* peroxidase which is oxidized two equivalents higher than the native enzyme. CcP-II represents compound II of the enzyme, oxidized one equivalent above the native enzyme. Fe^{2+} and Fe^{3+} represent ferrous and ferric iron in either cytochrome *c*, the natural substrate, or in small inorganic complexes such as the dicyano-bis(1,10 phenanthroline) complex used in this study.

When the ferrous complex is present in excess over the enzyme species, two pseudo-first-order rate constants can be defined for the reduction of compound I to the native enzyme via the mechanism shown in Scheme I.

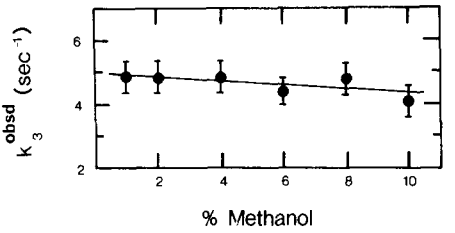
$$k_2^{obsd} = k_2^{app}[Fe^{2+}] \quad (1)$$

$$k_3^{obsd} = k_3^{app}[Fe^{2+}] \quad (2)$$

The method used to determine k_2^{obsd} and k_3^{obsd} from the time rate of change of absorbance values has been previously described [1]. Since the spectra of compounds I and II are similar, the reduction of compounds I and II was monitored near 424 nm, the maximum in the difference spectrum between the oxidized intermediates and the native enzyme [3]. The reduction of compound I to compound II has a relatively small amplitude in the semilogrithmic plots of absorbance versus time used to evaluate the pseudo-first-order rate constants. Consequently, k_2^{obsd} is subject to larger experimental error than k_3^{obsd}, the rate constant for the reduction of compound II to the native enzyme [1].

The effect of methanol concentration on the observed rate constants was determined. Fig. 1 shows values of k_3^{obsd} as a function of methanol concentration over the concentration range employed in this study. There is a slight decrease in k_3^{obsd} with increasing methanol. However, the decrease is small and within the experimental variation of k_3^{obsd} determined in replicate experiments. The values of k_3^{obsd} have a standard error of about ±15% in the pH range 3.5—7.5. Outside this region, the standard error is larger, primarily due to the instability of the enzyme at higher and lower pH.

In the limited concentration range available for study, the observed rate constants obey Eqns 1 and 2. No saturation is observed. The apparent bimolecular rate constants were evaluated from plots of the observed pseudo-first-



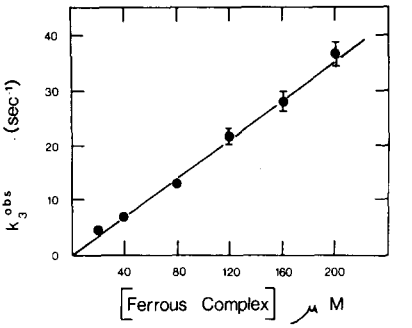


Fig. 1. The effect of methanol concentration on k_3^{obsd}. Experimental conditions: pH 4.1 buffer, 0.01 M acetate with varying amounts of methanol as indicated. Ionic strength was adjusted to 0.1 M with KNO_3. Cytochrome *c* peroxidase concentration, 0.49 μM; dicyano-bis(1,10 phenanthroline) iron(II) concentration, 20 μM.

Fig. 2. The effect of dicyano-bis(1,10 phenanthroline) iron(II) concentration on k_3^{obsd}. Same buffer as for Fig. 1 with 10% methanol. Cytochrome *c* peroxidase concentration, 0.49 μM.

order rate constants versus concentration of the ferrous complex, such as shown in Fig. 2.

The logarithms of the apparent bimolecular rate constants are plotted as a function of pH in Fig. 3. Note that k_2^{app} is multiplied by a factor of ten to offset it from k_3^{app}. Values for k_2^{app} were only determined between pH 3.5 and 6.5. Outside this region, the small amplitude of the reaction was obscured either by denaturation of the enzyme or the loss of the ferrous complex by precipitation from solution.

Values of k_3^{app} could be determined to pH 2.5 before the denaturation became fast enough to interfere. At pH 8, k_3^{app} has its smallest value and the loss of the iron complex from solution interfered with determination of the rate constant, causing a relatively large standard error at this pH. At pH 9 and

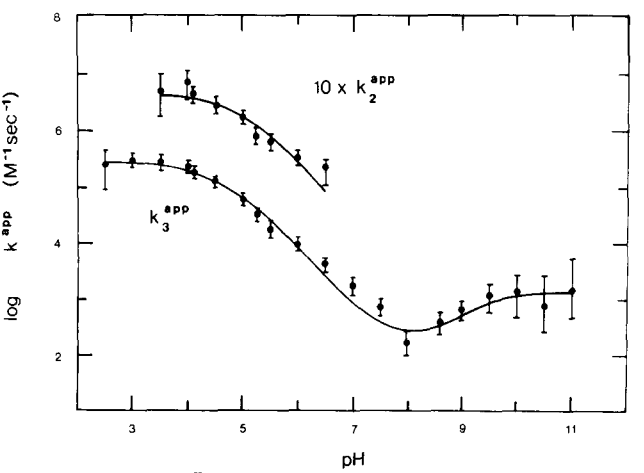


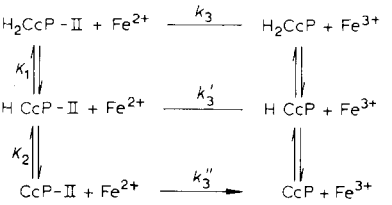
Fig. 3. Plot of log k_3^{app} and log k_3^{app} as a function of pH. Note the values of k_3^{app} have been multiplied by a factor of ten to offset them from the k_3^{app} values. The solid lines are calculated from a mechanism discussed in the text.

above, the denaturation of the enzyme causes a rapid endogenous reduction of the active sites in compounds I and II. Addition of the ferrous complex increases the rate of reduction. Values of k_3^{app} were calculated using the rate increase as a function of concentration but they are subject to large standard errors and the data are considerably less certain than in the pH region where the enzyme is stable.

Discussion

Dicyano-bis(1,10 phenanthroline) iron(II) is a very weak base and is not protonated in the pH region investigated [4]. The variation of the rate constants with pH must be due to the influence of the enzyme. A minimum of two ionizable enzyme groups which can influence the electron transfer rate must be postulated to explain the pH dependence of k_3^{app} between pH 2.5 and 11.

SCHEME II



Using the pH-independent rate constants, k_i, and the acid dissociation constants, K_i, defined in Scheme II, the variation of k_3^{app} with H^+ concentration is given by Eqn 3.

$$k_3^{app} = \frac{k_3 + k_3' K_1/[H^+] + k_3'' K_1 K_2/[H^+]^2}{1 + K_1/[H^+] + K_1 K_2/[H^+]^2} \quad (3)$$

The nonlinear least-squares curve fitting program, KINET, was used to evaluate the parameters in Eqn 3. Table I lists the parameters along with estimates of their standard errors.

In the pH region where k_2^{app} could be measured, it has values approximately three times larger than k_3^{app}. This is in accord with the study of the

TABLE I

RATE AND EQUILIBRIUM CONSTANTS

The values were obtained from fitting k_2^{app} and k_3^{app} to Scheme II. Conditions, 0.1 M ionic strength, 25°C, all k_i values in $M^{-1} \cdot s^{-1}$. All K_i values in M.

k_2^{app}		k_3^{app}	
k_2	$(4.6 \pm 1.1) \times 10^5$	k_3	$(2.9 \pm 0.4) \times 10^5$
		k_3'	$(1.8 \pm 0.4) \times 10^2$
		k_3''	$(2 \pm 2) \times 10^3$
K_1	$(1.5 \pm 0.6) \times 10^{-5}$	K_1	$(2.9 \pm 0.7) \times 10^{-5}$
		K_2	$(5 \pm 5) \times 10^{-10}$

reduction of compounds I and II by ferrocyanide where k_2^{app} was between two and three times larger than k_3^{app} at all conditions of pH and ionic strength investigated [1]. The values of the parameters obtained by fitting k_2^{app} to an equation analogous to Eqn 3 are also given in Table I. The solid lines in Fig. 3 were calculated using Eqn 3 and the parameters given in Table I.

This investigation has established that an ionizable group in cytochrome *c* peroxidase with a pK_a value of 4.5 ± 0.1 at 0.1 M ionic strength influences the electron transfer rate from dicyano-bis(1,10 phenanthroline) iron(II) to the oxidized site in compound II and probably to the oxidized site in compound I as well. The electron transfer is most rapid when the group is protonated, decreasing over three orders of magnitude when the proton dissociates. The identity of the group is not known, but the pK_a value suggests a carboxylic acid.

The instability of the enzyme above pH 8 and the resulting large errors in k_3^{app} make it difficult to be confident about the involvement of a second ionizable group in the electron transfer reaction. The pH dependence of k_3^{app} in the alkaline pH region could be a reflection of the electron transfer rates for a number of different enzyme conformations as the enzyme begins to denature. Unpublished observations on the denaturation of cytochrome *c* peroxidase at alkaline pH indicate that the denaturation is a multiphasic process.

Comparison of the rate of reduction of compound II by dicyano-bis(1,10 phenanthroline) iron(II) and by ferrocyanide [1] at 0.1 M ionic strength indicates that k_3^{app} is the same, within experimental error, for both ferrous complexes between pH 5.25 and 9.5. This was quite unexpected in view of the differences in net charge, redox potential, and electron self-exchange rates of the two complexes [5]. Below pH 5.25, the isoelectric point of the enzyme [6] the electron transfer rate from ferrocyanide is faster than that from the dicyano-bis(1,10 phenanthroline) complex. This is probably a reflection of the electrostatic attraction between the positively charged protein and the negatively charged ferrocyanide ion.

The close agreement of the electron transfer rates from the two ferrous complexes above pH 5.25 suggests both complexes may interact with the enzyme in the same manner regardless of the differences in their net charge. The agreement also suggests that both rates are influenced by the enzyme group with pK_a of 4.5. Therefore, the conclusion reached in the previous study must be modified to take into account the influence of the ionizable group on the electron transfer rate.

Preliminary investigation of the ionic strength dependence of the electron transfer rate between dicyano-bis(1,10 phenanthroline) iron(II) and compound II indicates that there is a substantial secondary kinetic salt effect on the apparent rate constants. This finding will also have to be taken into account in the interpretation of the ferrocyanide data.

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