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OXIDATION-REDUCTION POTENTIAL MEASUREMENTS OF CYTOCHROME c PEROXIDASE AND pH DEPENDENT SPECTRAL TRANSITIONS IN THE FERROUS ENZYME

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Summary

The redox potential of the ferrous/ferric couple in cytochrome c peroxidase has been measured as a function of pH between pH 4.5 and 8. The redox potential decreases linearly as a function of pH between pH 4.5 and 7 with a slope of $-57 \pm 2$ mV per pH unit. Above pH 7, there is a positive inflection in the midpoint potential versus pH plot attributed to an ionizable group in the ferrous enzyme with a $pK_a$ of $7.6 \pm 0.1$. The midpoint potential at pH 7 is $-0.194$ V relative to the standard hydrogen electrode at 25°C.

Ferrocytochrome c peroxidase undergoes a reversible spectral transition as a function of pH. Below pH 7, the enzyme has a spectrum typical of high spin ferroheme proteins while above pH 8, the spectrum is typical of low spin ferroheme proteins. The transition is caused by a co-operative, two proton ionization with an apparent $pK_a$ of $7.7 \pm 0.2$. Two other single proton ionizations cause minor perturbations to the spectrum of ferrocytochrome c peroxidase. One has a $pK_a$ of $5.7 \pm 0.2$ while the second has a $pK_a$ of $9.4 \pm 0.2$.

Introduction

Cytochrome c peroxidase (ferrocytochrome c:hydrogen peroxide oxidoreductase, EC 1.11.1.5) is a hemeprotein which catalyzes the oxidation of ferrocytochrome c by hydrogen peroxide [1,2]. The reaction mechanism involves a two-electron oxidation of the native ferric enzyme by hydrogen peroxide to form an oxidized intermediate [1]. The oxidized intermediate is reduced back to the native enzyme via two one-electron steps by ferrocytochrome c [2]. The reactions which occur at the heme site of the native ferric enzyme are influenced by an ionization with an apparent $pK_a$ of 5.5 at 0.1 M ionic strength [3–6]. The enzyme is reactive only when the ionizable group is unprotonated [5,6]. The ionization of the $pK_a$ 5.5 group also causes a slight perturbation of
the Soret band in the absorption spectrum (Conroy, C. and Erman, J., unpublished data). To further characterized the influence of the pKₐ 5.5 group on the properties of cytochrome c peroxidase, the oxidation-reduction potential of the ferric/ferrous couple has been measured as a function of pH. In addition, the pH dependence of the absorption spectrum of ferrocytochrome c peroxidase has been investigated.

Materials and Methods

Cytochrome c peroxidase was isolated from commercial baker’s yeast according to the method of Yonetani [7,8]. The enzyme was crystallized at least twice from distilled, deionized water and stored at −10°C until used. Enzyme solutions were prepared by dissolving the crystals in the desired solvent, centrifuged at 10 000 × g for 15 min to remove any insoluble material. The enzyme concentration was determined spectrophotometrically using an extinction coefficient of 95 mM⁻¹·cm⁻¹ at 408 nm [2], the maximum of the Soret band.

Methyl viologen was purchased from Sigma Chemical Company. A preliminary spectrum of a 0.5 M solution of this substance showed appreciable absorption between 280 and 300 nm. The methyl viologen was purified by repeated crystallization from hot acetone-methanol solution. Indicator-mediator dyes (neutral red, anthroquinone 2-sulfonate, 2-hydroxynaphthaquinone indigo carmine, and nile blue) were purchased from Sargent Welch or Aldrich and used without further purification. All other chemicals used were reagent grade.

Oxidation-reduction titrations were generally performed with 3.0 ml of solution. The enzyme concentration ranged between 10 and 50 μM and the solutions contained a suitable buffer, 0.5 to 2 mM methyl viologen and potassium ferrocyanide and an appropriate mediator dye present at 2.5% of the enzyme concentration. No interaction between the enzyme and reagents were detected under these conditions. Acetate buffers were used between pH 4.5 and 6 while phosphate buffers were used between pH 6 and 8.

Helium gas was purged of oxygen by passage through a 30 cm quartz tube packed with copper gauze maintained at 600°C and then humidified by bubbling through water before being passed into the cell. A positive gas pressure was maintained in the cell at all times during the titration to exclude oxygen. Reduction of the enzyme was accomplished by formation of the methyl viologen cation radical at the platinum grid with the potential between the grid and counter electrode maintained at −0.65 V. Residual oxygen was removed from the enzyme solution during the first phase of the reduction by the viologen cation, so prior extensive deaeration of the enzyme solution in the cell was not necessary.
Oxidation of the enzyme was accomplished by the formation of ferricyanide from ferrocyanide at the platinum grid with the electrochemical potential maintained at +0.4 V.

Potential measurements were made with the potential mode of the PAR potentiostat between the platinum wire and saturated calomel electrode when the enzyme couple was between 20 and 80% reduced. In the presence of suitable mediator dyes, the potential usually stabilized within 3 min and was stable to within 0.5 mV for at least 30 min. The ratio of oxidized to reduced enzyme was determined from the absorbance at 438 nm. Generally 4 to 5 potential-absorbance measurements were made in the 20–80% reduction range for both reduction and oxidation of the enzyme. Potentials obtained from the reductive phase and the oxidative phase of the titrations agreed to within 1 mV. It was possible to cycle the system through as many as five oxidation-reduction cycles with no change in the measured potentials. The pH of the solution was measured both before and after the experiments to ensure that no change occurred during the experiment.

An alternate titration procedure was found to give reliable values for the midpoint potential, although not routinely used. This technique involved poising the enzyme couple at 50% oxidized-50% reduced, then varying the pH by the addition of deaerated acid or base. The measured potential usually stabilized within 1 min after reaching the final pH. Potentials so obtained were in excellent agreement with those obtained by the first titration method. This procedure was especially useful above pH 7 where spectral alterations of the ferrous enzyme made spectrophotometric determinations of the oxidized/reduced ratio difficult.

The pH dependent difference spectra of the ferrous enzyme were measured with the absorption mode of a JASCO CD/ORD spectrophotometer. The sample cuvette was constructed to permit stirring of the contents, measurement of the pH, provide access for two microburette tips through which acid or base could be admitted to the cell, and to provide a port for passing helium gas over the surface of the solution. Identical enzyme solutions were placed in both a sealed reference cuvette and the sample cuvette. The deoxygenated samples were reduced with identical aliquots of dithionite and the base line recorded. Increments of acid or base were then added to the sample cuvette and the pH of the sample, along with the difference spectrum were recorded. The volume of acid or base added was negligible and no corrections were required due to dilution of the sample solution. The absolute spectrum of the ferrous enzyme was determined either by reducing the enzyme in a buffer at the appropriate pH or by reducing the enzyme in a 0.1 M KNO₃ solution and adjusting the pH by adding deoxygenated acid or base.

Results

At all pH values studied, the oxidation-reduction potential of cytochrome c peroxidase obeyed Eqn. 1

\[ E_h = E_m + \frac{2.3 \, RT}{nF} \log([\text{Ox}]/[\text{Red}]) \] (1)
The results of a typical potentiometric titration of the enzyme are shown in Fig. 1. The slope of the logarithm of the ratio of oxidized to reduced enzyme versus the measured potential was close to 0.06 indicating the process to be mono-electronic.

Potential measurements at pH values below 4.5 were not obtained due to precipitation of the enzyme under the conditions of the experiment. Above pH 8 the potentials were observed to drift slowly at first, then more rapidly toward positive values with no stabilization apparent after 30 min. This behavior is in marked contrast to potentials below pH 8 where the measured values always drifted toward more negative values as equilibrium was being established. It is known that the ferric form of cytochrome c peroxidase undergoes a series of complex transitions above pH 8 which occur over a period of many hours (Dhaliwal, B. and Erman, J., unpublished data). No equilibrium potentials were determined above pH 8.

The midpoint potential of cytochrome c peroxidase was found to vary with pH from a value of $-52$ mV at pH 4.5 to $-202$ mV at pH 8 as shown in Fig. 2. Each value shown in Fig. 2 is the average of 2–5 separate determinations with an average standard deviation of 3 mV. Between pH 4.5 and 7 the midpoint potential decreases linearly as a function of pH with a slope of $-57 \pm 2$ mV per pH unit. Above pH 7, there is a positive inflection in the slope of the $E_m$ versus pH plot.

The spectrum of ferrous cytochrome c peroxidase was investigated between pH 4.5 and 12. In the acidic pH region, there is a small decrease, about 5%, in the Soret maximum as the pH is lowered from about pH 6.5 to 5 (Fig. 3). Above pH 6.5, large spectral alterations are observed in both the visible and Soret regions (Fig. 4). Above about pH 7.5, the absorbance of the enzyme is dependent upon time. The time dependence appears complex, with the major change complete within the time required for pH adjustment, usually a few seconds. However there are small absorbance changes which occur over the period of several minutes to hours. The spectra shown in Fig. 4 at pH 8.55 and 10.5 were recorded immediately after adjusting the pH to the reported values. The time dependence of the spectral changes were not studied in detail but...
Fig. 3. Spectrophotometric titration of ferrous cytochrome c peroxidase in acidic media. Difference spectra as a function of pH were taken relative to a reference sample at pH 6.70. The difference spectra were recorded at pH 6.50, 6.31, 6.05, 5.85, 5.59, 5.37, and 5.02 in order of increasing negative amplitude.

Fig. 4. Spectra of ferrocytochrome c peroxidase at pH 6.0, 8.55, and 10.5.

their existence suggests the spectral transitions in the alkaline region are not due solely to acid-base equilibria, but that the protein undergoes conformational isomerization in this pH region.

The extinction coefficient of ferrocytochrome c peroxidase at 444 nm is shown in Fig. 5 as a function of pH. The extinction coefficient at 444 nm is illustrated because the largest changes between pH 5 and 6.7 occur at this wavelength (Fig. 3). In addition, the extinction coefficient at 444 nm shows the sharp transition between pH 7 and 8.5 in which the spectrum changes from one which has the Soret maximum at 438 nm and a single maximum in the visible region at 559 nm to a spectrum with the Soret maximum at 426 nm and the

Fig. 5. The variation of the extinction coefficient at 444 nm as a function of pH. The solid line was calculated by a mechanism discussed in the text involving three ionizations on the enzyme.

Fig. 6. A Hill plot of the transition centered near pH 7.7. Theta (θ) is the fraction of enzyme in the acidic form and was calculated from the absorbance at 589 nm.
α and β bands at 562 and 530 nm respectively. A third transition is apparent above pH 8.5, characterized by an increase in intensity at the Soret maximum and a decrease in intensity in the α and β bands.

The spectral transition between pH 7 and 8.5 appears to be steeper than expected assuming that the ionization of a single ionizable group is responsible for the change. A Hill plot [9] of the change in absorbance at 589 nm as a function of pH is shown in Fig. 6. At this wavelength, the transitions between 5 and 6.5 and above 9 do not contribute significantly to the change in absorbance. The slope of the plot in Fig. 6 is 1.90 ± 0.16 with an apparent pKₐ of 7.7 ± 0.2. This data suggests that the transition centered at pH 7.7 involves a co-operative, two proton ionization.

The spectral transitions are fully reversible between pH 4.5 and 10. Fitting the extinction coefficients at several wavelengths to a mechanism involving three ionizations on the enzyme including the co-operative, two proton ionization, gives apparent pKₐ values of 5.7 ± 0.2, 7.7 ± 0.2 and 9.4 ± 0.2. The solid line in Fig. 5 was calculated based on the three ionization mechanism.

Discussion

The midpoint potential of the ferrous/ferric cytochrome c peroxidase couple at pH 7 is −0.194 V versus the standard hydrogen electrode. This value is about midway between the extremes found for peroxidase, the most negative being horseradish peroxidase at −0.270 V [10] and the least negative being turnip peroxidase P₇ at −0.109 V [11]. The gradation of the redox potential in the peroxidases (and in heme proteins in general) has been attributed to the changing character of the heme binding site [12]. In heme proteins with the more negative potentials, it has been suggested a carboxylate group stabilizes the ferric state relative to the ferrous state [13].

The pH dependence of the redox potential in horseradish peroxidase and the turnip peroxidases P₁ and P₇ is similar in that the potential decreases with increasing pH at low pH, attains a plateau region near neutrality, and then decreases with increasing pH again at high pH [11]. The simplest mechanism to explain this behavior is that at low pH, the ferrous form of the peroxidases bind one more proton than the ferric form of the enzymes. In order to reduce the ferric enzyme both a proton and an electron are required. At the pH of the positive inflection in the redox potential versus pH curve, the ferrous enzyme undergoes an acid-base ionization which is not present in the ferric enzyme. At pH values above this ionization, the ferrous and ferric forms of the enzyme bind the same number of protons. Reduction only requires an electron and the redox potential is independent of pH in this pH region. At still higher pH, the ferric enzyme has an acid-base ionization which is not present in the ferrous form. Again the ferrous form of the enzyme binds one more proton than the ferric form and both a proton and an electron are required for reduction. The proton requirement is reflected in the decreasing value of the redox potential with increasing pH at high pH. For horseradish peroxidase and turnip peroxidase P₁, the pKₐ of the oxidized enzyme group corresponds to the spectroscopically observed ‘acid-alkaline’ transition in which the ferric enzyme undergoes a
spin-state change from predominantly high spin at neutral pH to low spin at alkaline pH [10,11].

Ferric cytochrome c peroxidase undergoes a series of complex spectral transformations above pH 8. Stopped-flow studies indicate the 'acid-alkaline' transition in cytochrome c peroxidase has a pKₐ of 9.8. The alkaline form is unstable and isomerizes to a largely unfolded state (Dhaliwal, B. and Erman, J. unpublished data). As a consequence only the acidic limb of the redox potential curve can be observed for cytochrome c peroxidase. Between pH 4.5 and 7, both an electron and a proton are required for the reduction of ferric cytochrome c peroxidases and the redox potential decreases as the pH increases with a slope of $-57 \pm 2$ mV/pH. The positive curvature in the plot of $E_m$ versus pH near 7.5 is due to an ionization in the reduced form of the enzyme and most likely corresponds to the spectroscopically observed ionization, Figs. 4 and 5. The redox potential measurements could not be made at high enough pH values to distinguish whether the inflection in the redox potential versus pH curves is due to a single or double proton ionization. An ionization involving a single proton would cause the redox potential to become independent of pH above pH 8, while a double proton ionization would cause the redox potential to increase with increasing pH above pH 8. Although stable potentials were not obtained above pH 8, they were observed to drift toward more positive values. Fitting the midpoint potential to the mechanism involving the co-operative, two proton ionization in the reduced enzyme gives an apparent pKₐ of 7.6 ± 0.1 in agreement with the values obtained from the spectroscopic measurements.

In the ferric enzyme, there is an ionization with a pKₐ of 5.5 which controls the reaction of hydrogen peroxide with the enzyme and also influences the binding of small ligands such as fluoride and cyanide to the heme site [3–6]. Since no inflection is observed in the redox potential near pH 5.5, this implies the existence of a group in the ferrous form of the enzyme with a pKₐ near 5.5. The spectral titration of the ferrous enzyme, Figs. 3 to 5 demonstrate the presence of this group. The small perturbation of the ferrous spectrum by this group is analogous to the perturbation in the ferric enzyme (Conroy, C. and Erman, J., unpublished data).

The most striking feature of cytochrome c peroxidase discovered in this investigation is the co-operative ionization which converts the ferrous enzyme from a species which has a spectrum characteristic of a high spin ferrous heme protein to one which has a spectrum analogous to low spin heme proteins [12]. We suggest that at neutral pH, the iron atom of ferrous cytochrome c peroxidase is a five-coordinantate species. During the transition at pH 7.7 an amino acid residue near the heme iron deprotonates and, in its basic form, coordinates with the heme iron, forming the low-spin species. Ferrous turnip peroxidase P₇ undergoes a similar spectral transition with an apparent pKₐ of 8.2 [11]. However the ionization is a normal, single proton process (see Fig. 6, reference 11).

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