# pH-Dependent Spectral and Kinetic Properties of Cytochrome *c* Peroxidase: Comparison of Freshly Isolated and Stored Enzyme†

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ABSTRACT: The effect of long-term storage on the electronic absorption spectrum and the kinetic properties of cytochrome *c* peroxidase has been investigated. No detectable differences were observed between freshly isolated enzyme and enzyme stored below −20 °C, in the crystalline state, for up to 41 months. The electronic absorption spectrum and the rate of the enzyme–hydrogen peroxide reaction are essentially independent of pH in 0.1 M potassium phosphate buffers for both fresh and stored enzyme. In buffers containing $KNO_3$, the absorption spectrum and the kinetic properties of both fresh and stored enzyme vary with pH, consistent with the titration of an ionizable group with an apparent $pK_a$ of 5.5 ± 0.1. The differences between phosphate- and nitrate-containing buffers are attributed to specific ion effects. In $KNO_3$-containing buffers, the high-pH form of the enzyme reacts rapidly with hydrogen peroxide while the low-pH form is unreactive. Evidence is presented which indicates that both the low-pH and high-pH forms of the enzyme in $KNO_3$-containing buffers are 5-coordinate, high-spin Fe(III) species.

Cytochrome *c* peroxidase (CcP)[1] was discovered nearly 50 years ago (Altschul et al., 1940) but received scant attention until Yonetani and co-workers developed a simple isolation procedure yielding highly purified, crystalline enzyme (Yonetani & Ray, 1965; Yonetani et al., 1966). Interest in the enzyme has accelerated in recent years due to the elucidation of the three-dimensional structure of the enzyme (Poulos et al., 1980; Finzel et al., 1984) and by the development of cloning systems for the production of mutant CcP molecules (Goodin et al., 1986; Fishel et al., 1987). Along with the increased interest, a number of apparently conflicting observations have been reported about the purity, stability, and spectroscopic and kinetic properties of the enzyme. While the Yonetani isolation procedure generally gives enzyme preparations with a purity index (PZ) of 1.2–1.3, more recent methods, using acetate buffers and lower pH conditions, have produced enzyme preparations with PZ values near 1.5 (Nelson et al., 1977; English et al., 1986). The higher PZ values do not appear to be related to significantly more homogeneous enzyme preparations, suggesting that the variation in PZ is due to differences in the absorption spectra of the various preparations. Resonance Raman studies indicate enzyme preparations with PZ values between 1.25 and 1.30 have 5-coordinate, high-spin heme (Dasgupta et al., 1989) while enzyme preparations with PZ values near 1.5 are mixtures of 5-coordinate and 6-coordinate high-spin forms (Smulevich et al., 1986).

The original observations that long-term storage, or aging, of CcP may alter the enzyme's properties were made by Smulevich et al. (1986) on the basis of resonance Raman and IR studies of CcP. Their fresh enzyme samples were predominantly 6-coordinate, high-spin species with some 5-coordinate, high-spin form in 10 mM phosphate buffer, pH 7. Moderately aged CcP was primarily 5-coordinate, high-spin with a significant fraction of 6-coordinate, low-spin species and essentially no 6-coordinate, high-spin forms. The presence of the 6-coordinate, high-spin form in freshly isolated CcP and the development of the 6-coordinate, low-spin species during storage have been attributed to the binding of acetate during isolation and storage (Smulevich et al., 1989).

Yonetani and Anni (1987) and Dasgupta et al. (1989) have also noted changes in the spectroscopic properties of CcP during prolonged storage. Freshly isolated CcP, prepared by the Yonetani method, with PZ values between 1.25 and 1.30, is entirely 5-coordinate, high-spin between pH 4.3 and 7.0. Storage of enzyme solutions in 1 M phosphate buffers, pH 5.5–6.0, for a week at 4 °C or for several months under liquid nitrogen produced enzyme with PZ values greater than 1.30. Resonance Raman spectroscopy showed that these aged samples were mixtures of 5- and 6-coordinate, high-spin species at pH 4.3 and, in addition, contained some 6-coordinate, low-spin forms at pH 7.0. The studies of Smulevich et al. (1986, 1989), Yonetani and Anni (1987), and Dasgupta et al. (1989) indicate that the coordination state of the heme iron in CcP can depend upon the isolation procedure, storage conditions, and buffer composition.

Yonetani and Anni (1987) report that the electronic absorption spectrum of freshly isolated CcP is invariant between pH 4 and 8 while the absorption spectrum of aged CcP varies with pH. The spectrum of aged CcP shows an increase in absorptivity at the Soret maximum, a decrease between 360 and 380 nm, and a shift of the charge transfer band observed at 645 nm in 5-coordinate, high-spin CcP to 620 nm in the 6-coordinate, high-spin form. The changes are most pronounced at low pH. We have previously reported small changes in the absorption spectrum of CcP as a function of pH (Conroy & Erman, 1978), and Yonetani and Anni have suggested that this pH dependence is due to the presence of aged CcP.

In addition to the spectroscopic properties, the kinetic properties of the enzyme can show significant differences. Our laboratory reported that the reaction between hydrogen per-

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[1] Abbreviations: CcP, bakers' yeast cytochrome *c* peroxidase; PZ, purity index, ratio of the absorbance at the Soret maximum to the maximum absorbance of the protein near 280 nm; DEAE, diethylaminoethyl; KP, potassium phosphate buffer; KOAc, potassium acetate buffer; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance.

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oxide and CcP was dependent upon pH (Loo & Erman, 1975). An apparent enzyme ionization, with a $pK_a$ of 5.5, influences the reaction rate. The high-pH form of the enzyme reacted rapidly with hydrogen peroxide while the low-pH form has no reactivity within experimental error. Balny et al. (1987) report that the reaction rate between hydrogen peroxide and CcP is independent of pH between pH 4.5 and 8.

When the reports on the effect of storage upon the spectroscopic properties of CcP first appeared in the literature, we were quite concerned since all of our previous work had been carried out using enzyme preparations which were stored below −20 °C in the crystalline state. The substantial differences in the kinetic properties reported by Balny et al. (1987) and ourselves were also puzzling and prompted us to investigate the effect of storage on our enzyme preparations. In this report, we describe spectroscopic and kinetic studies using freshly isolated CcP and enzyme samples stored below −20 °C for 15–41 months in the crystalline state. We can detect no significant differences between fresh and stored enzyme samples. We conclude that all of our previous studies, including the pH dependence of the electronic absorption and the resonance Raman and NMR spectra as well as the rate of the reaction with hydrogen peroxide, are properties of the native, 5-coordinate, high-spin enzyme. We have discovered that many of the differences reported in the literature can be ascribed to specific-ion effects and that there are significant differences in the properties of the enzyme in solutions containing nitrate as the supporting electrolyte compared to those with high phosphate concentrations.

## Materials and Methods

*Isolation of CcP.* Since the isolation procedure may play a role in determining the properties of the enzyme, our isolation procedure is described in detail. The procedure is a modification of that of Yonetani and Ray (1965). Ethyl acetate (3 L) is added to 50 lb of fresh, compressed bakers' yeast (Red Star, Universal Food Products, Milwaukee, WI) and mixed to give a homogeneous paste which is maintained at 4 °C overnight. Distilled water is added to the lysed yeast to bring the total volume to 30 L; the mixture is stirred at room temperature for 1–2 h and then centrifuged at 7500*g* for 15 min in 3-L batches. The supernatant (about 20 L) is dialyzed overnight, adjusted to pH 6.5, filtered, and applied to two 5 × 50 cm columns of DEAE-cellulose equilibrated in 5 mM potassium phosphate buffer, pH 6.5. The sample is eluted with 0.5 M potassium phosphate, pH 6. CcP elutes as a dark, reddish brown band, generally in a total volume of about 1.5 L. The sample is dialyzed overnight against 15 L of distilled $H_2O$ and applied to a second DEAE-cellulose column (5 × 50 cm) equilibrated in 5 mM potassium phosphate, pH 6.0. This column is washed with 2 L of 50 mM potassium phosphate, pH 6.0, and a sufficient volume of 100 mM potassium phosphate, pH 6.0, is used to spread the reddish brown band containing the CcP to the bottom of the column. The enzyme is eluted with 0.5 M potassium phosphate, pH 6.0. About 500 mL of sample is collected. The sample is dialyzed against distilled water and then concentrated to about 50 mL on a small DEAE column. The concentrated sample is dialyzed against several changes of deionized–distilled water and seeded with CcP crystals. The 1× crystals are collected after 3–5 days, redissolved in a minimal amount of 0.5 M potassium phosphate buffer, pH 6, and again dialyzed against deionized–distilled water. Crystallization is spontaneous and complete in about 3 days. The 2× crystals are collected by centrifugation, the mother liquor is decanted, and the 2× crystalline CcP is stored below −20 °C in tightly sealed tubes.

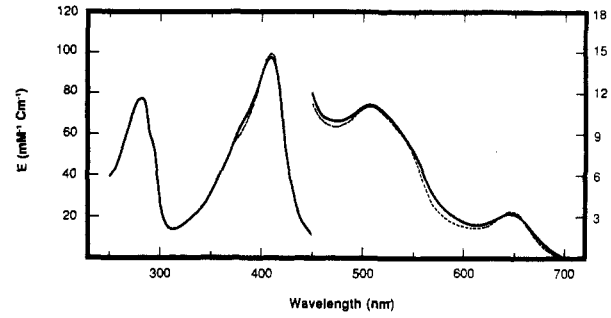


FIGURE 1: UV/visible spectra of freshly isolated CcP in pH 7.0, 10 mM potassium phosphate buffer with $KNO_3$ to 0.1 M ionic strength (solid line) and in pH 4.5, 10 mM potassium acetate buffer with $KNO_3$ to 0.1 M ionic strength (dashed line).

For the fresh enzyme used in this study, the undissolved, 2× crystals were stored at 4 °C and all experiments completed within 7 days of collection. After completion of the studies on the fresh enzyme, the undissolved 2× crystals were stored below −20 °C for 15–17 months and then reexamined to determine whether storage altered the properties of the enzyme.

Electronic absorption spectra and difference spectra were obtained by using a Cary Model 219 UV/visible spectrophotometer. Stopped-flow studies were performed with either a Dionex Model D-110 stopped-flow spectrometer or a Hi-Tech Instruments SF/PQ53 stopped-flow spectrofluorometer. All kinetic runs were carried out at 25 °C, under pseudo-first-order conditions as previously described (Loo & Erman, 1975).

## Results

*Spectral Properties of Freshly Isolated and Stored CcP.* To investigate the effect of storage on the electronic absorption spectrum of CcP, we determined the spectral properties of freshly isolated CcP (never frozen) for five different preparations during the fall of 1987. After completion of the studies on the fresh enzyme, the unused portions of four of the crystalline samples were frozen and stored below −20 °C for 15 – 17 months when their properties were reexamined. We also determined the spectral properties of a sample of CcP which had been stored in the crystalline state for 41 months.

Five different buffers were chosen for the spectral studies: (1) pH 7.0, 10 mM potassium phosphate with sufficient $KNO_3$ to adjust the ionic strength to 0.1 M (KP/$KNO_3$); (2) pH 7.0, 100 mM potassium phosphate (KP); (3) 0.1 M $KNO_3$; (4) pH 4.5, 10 mM potassium acetate with sufficient $KNO_3$ to adjust the ionic strength to 0.10 M (KOAc/$KNO_3$); (5) pH 4.5, 100 mM potassium phosphate (KP). The buffers with $KNO_3$ were chosen since most of our previous work on the pH-dependent properties of CcP was carried out in these buffers. The 100 mM potassium phosphate buffers were chosen since Yonetani and co-workers report that the spectral and kinetic properties of CcP are pH invariant in 100 mM phosphate (Balny et al., 1987; Yonetani & Anni, 1987; Dasgupta et al., 1989).

The spectrum of freshly isolated CcP in pH 7.0 KP/$KNO_3$ buffer is shown in Figure 1. This spectrum is identical with that obtained in pH 7.0 KP buffer. Average values of the wavelengths of maximum absorbance and the absorptivities for the five fresh and the five stored samples of CcP are collected in Table I. Within experimental error, the spectra of freshly isolated and stored enzyme are identical at pH 7.0 in the two buffer systems.

We looked for changes in the shape of the absorption spectrum by determining absorptivity ratios at various wavelengths. The critical ratios for determining the presence of aged CcP are the ratio of the absorptivity at the Soret

Table I: Spectral Characteristics of Fresh and Stored CcP at pH 7.0[a]

| band | fresh $\lambda_{max}$ (nm) | fresh $\epsilon^c$ ($mM^{-1}$ $cm^{-1}$) | stored[b] $\lambda_{max}$ (nm) | stored[b] $\epsilon^c$ ($mM^{-1}$ $cm^{-1}$) |
|---|---|---|---|---|
| protein | 282 | 82 ± 3 | 282 | 83 ± 8 |
| soret | 408 | 99 ± 3[d] | 409 | 97 ± 3[d] |
| CT1[e] | 507 | 11.4 ± 0.2 | 508 | 11.2 ± 0.7 |
| CT2[e] | 646 | 3.4 ± 0.1 | 647 | 3.4 ± 0.2 |

[a] Data acquired for five different fresh and five different stored enzyme samples in two pH 7.0 buffers, 100 mM potassium phosphate and 10 mM potassium phosphate with $KNO_3$, to adjust the ionic strength to 0.10 M. Wavelength accuracy is ±1 nm. [b] Crystalline enzyme was stored below −20 °C for 15–41 months. [c] Absorptivities were determined relative to the Soret maximum and are the average of 10 determinations. [d] Determined by the pyridine hemochromogen method for two preparations. [e] Charge transfer bands.

Table II: Ratio of Absorptivities (nm) at Conformationally Sensitive Wavelengths for Fresh and Stored CcP

| pH | buffer[a] | conditions[b] | ratios of absorptivities 408/380[c] | ratios of absorptivities 620/647[d] |
|---|---|---|---|---|
| 7.0 | KP and KP/$KNO_3$ | fresh | 1.51 ± 0.03 (10)[e] | 0.73 ± 0.03 (10) |
| | | stored | 1.52 ± 0.04 (10) | 0.76 ± 0.05 (10) |
| 4.5 | KP | fresh | 1.54 ± 0.01 (5) | 0.76 ± 0.03 (5) |
| | | stored | 1.50 ± 0.03 (5) | 0.76 ± 0.02 (5) |
| 4.5 | KOAc/$KNO_3$ | fresh | 1.60 ± 0.06 (5) | 0.70 ± 0.04 (5) |
| | | stored | 1.60 ± 0.03 (5) | 0.73 ± 0.04 (5) |

[a] KP, 100 mM potassium phosphate; KP/$KNO_3$, 10 mM potassium phosphate with the ionic strength adjusted to 0.1 M with $KNO_3$; KOAc/$KNO_3$, 10 mM potassium acetate with the ionic strength adjusted to 0.1 M with $KNO_3$. [b] Fresh enzyme was used within 7 days of isolation and never frozen. Stored enzyme was stored in the crystalline state below −20 °C for 15–41 months. [c] The maximum absorptivities occurred at 408 ± 1 nm. [d] The maximum in the charge transfer band occurred at 647 ± 2 nm. [e] Values in parentheses refer to the number of determinations.

maximum to that in the shoulder at 380 nm (Figure 1) and the ratio of the absorptivity at 620 nm relative to that of the charge transfer band near 647 nm. Both of these ratios should increase with increasing amounts of aged CcP, and the increase should be most pronounced at acidic pH (Yonetani & Anni, 1987). Values of the ratios for fresh and stored enzyme at both pH 7.0 and pH 4.5 are collected in Table II.

Comparison of the values in Table II shows that storage of crystalline CcP for periods ranging between 15 and 41 months has absolutely no effect on the electronic absorption spectrum of CcP under the conditions investigated. The only statistically significant differences ($t$ test) in Table II were the absorptivity ratios in pH 4.5 KOAc/$KNO_3$ buffer in comparison to the other three buffer systems. When the fresh and stored enzyme data are averaged, the 408/380-nm absorptivity ratio in pH 4.5 KOAc/$KNO_3$ buffer has a slightly larger value (99% confidence level) and the 620/647-nm absorptivity ratio has a slightly lower value (95% confidence level). Since both fresh enzyme and stored enzyme show the same behavior, these small changes are not due to storage-related artifacts but must be due to buffer effects (see Discussion).

We have previously reported small changes in the spectrum of stored CcP as a function of pH in $KNO_3$-containing buffers (Conroy & Erman, 1978). We now demonstrate that freshly isolated CcP shows the same pH-dependent changes. The spectrum of freshly isolated CcP in pH 4.5 KOAc/$KNO_3$ buffer is shown in Figure 1. The spectrum acquired at pH 4.5 is nearly identical with that at pH 7.0 but does show small, systematic changes (i.e., an increase at the Soret maximum and a decrease at 380 nm). These changes are more readily observed by using difference spectroscopy, and the difference

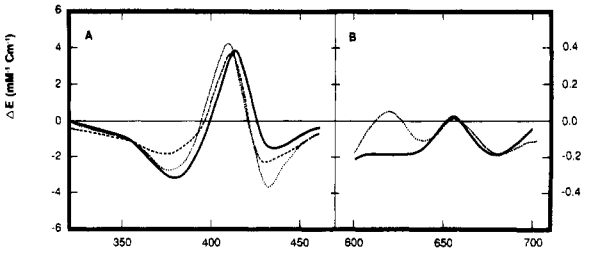


FIGURE 2: Difference spectra between low-pH and high-pH forms of CcP. (A) Soret region between 320 and 460 nm. (B) Long-wavelength region between 600 and 700 nm. Solid line, fresh CcP in 0.1 M $KNO_3$, pH 4.20 minus pH 7.25. Dashed line, stored CcP in 0.1 M $KNO_3$, pH 4.46 minus pH 7.03; data from Conroy and Erman (1978). Dotted line, fresh CcP in pH 4.50, 10 mM potassium acetate buffer with $KNO_3$ to 0.1 M ionic strength minus pH 7.00, 10 mM potassium phosphate buffer with $KNO_3$ to 0.1 M ionic strength.

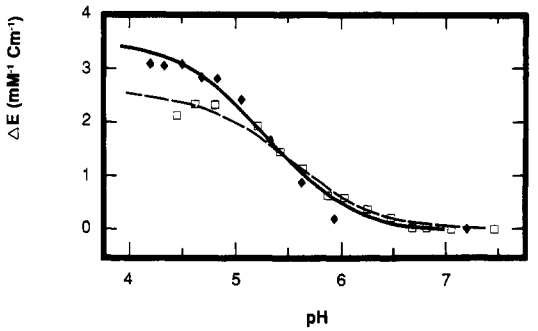


FIGURE 3: Change in the absorptivity of CcP at 415 nm as a function of pH. Solid diamonds, fresh CcP; open squares, stored CcP; data from Conroy and Erman (1978). Enzyme was dissolved in 0.1 M $KNO_3$ and pH adjusted with 0.1 M $HNO_3$ or 0.1 M KOH. The solid and dashed lines are calculated for the titration of an enzyme group with $pK_a$ of 5.4 ± 0.1 (fresh enzyme) and 5.5 ± 0.1 (stored enzyme).

spectrum between 320 and 460 nm and between 600 and 700 nm is shown in Figure 2. Included in Figure 2 are difference spectra for freshly isolated CcP and for stored enzyme obtained during pH titration studies of the spectral changes in 0.1 M $KNO_3$. Considering the small changes in absorptivity, the difference spectra in the Soret region are essentially identical. All three difference spectra show a positive peak near 410 nm and negative troughs near 380 and 430 nm. The magnitude of the amplitude changes is similar for both fresh and stored enzyme and is consistent with the changes in the 408/380-nm absorptivity ratios reported in Table II.

Between 600 and 700 nm, the difference spectra have very small amplitudes. In general, the absorptivity at acidic pH is less than the absorptivity at pH 7 in this region of the spectrum. It is important to note that there is no significant increase in the absorptivity at 620 nm at low pH when compared to the absorptivity near neutral pH (see also Figure 1). The pH dependence of the spectrum of freshly isolated CcP in 0.1 M $KNO_3$ solution was determined by difference spectroscopy (Figure 2). The absorptivity changes at 415 nm as a function of pH are shown in Figure 3 along with previously published data for stored enzyme (Conroy & Erman, 1978). The pH dependence of the absorbance changes is essentially identical for freshly isolated and stored enzyme in these solutions, again demonstrating that the spectral changes are not storage-related artifacts. The pH dependence of the absorptivity change at 415 nm is consistent with a mechanism involving a single ionization in the enzyme with an apparent $pK_a$ of 5.5 (see Discussion).

*Reaction between CcP and Hydrogen Peroxide.* We have previously shown that the rate of the reaction between hy-

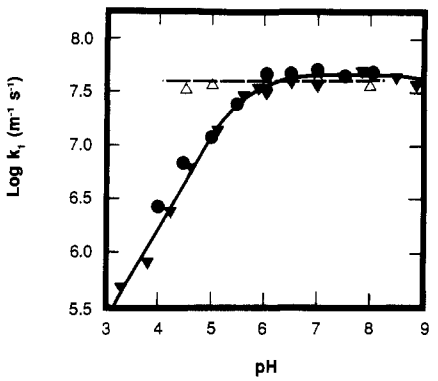


FIGURE 4: Plot of the log of the apparent bimolecular rate constant for the CcP–hydrogen peroxide reaction as a function of pH. Solid circles, fresh CcP in 10 mM acetate (pH 4–5.5) or 10 mM phosphate (pH 5.5–8) with $KNO_3$ added to adjust the ionic strength to 0.1 M. Solid inverted triangles, stored enzyme in the same buffer as above for fresh enzyme. Data from Loo and Erman (1975). Open triangles, stored enzyme in 0.1 M potassium phosphate buffers. The solid line was calculated by assuming CcP exists in high- and low-pH forms related by a $pK_a$ of 5.5 and that only the high-pH form reacts with hydrogen peroxide.

drogen peroxide and CcP forming CcP compound I is pH dependent in buffers using $KNO_3$ to adjust the ionic strength (Loo & Erman, 1975). In 0.1 M ionic strength buffers, an apparent $pK_a$ of 5.5 influences the rate. The high-pH form of the enzyme reacts rapidly with hydrogen peroxide, while the low-pH form had no detectable activity. These data were collected by using stored enzyme preparations. Experiments were carried out to determine whether freshly isolated CcP showed the same pH-dependent behavior. The data are shown in Figure 4. It is clear that the rate of the reaction between hydrogen peroxide and freshly isolated CcP is dependent upon pH in buffers using $KNO_3$ as supporting electrolyte. Furthermore, the data are within experimental error of our previously published results using stored enzyme (Loo & Erman, 1975).

Balny et al. (1987) reported that the hydrogen peroxide–CcP reaction is independent of pH. The major difference between our studies and those of Balny et al. appears to be the choice of buffer. Balny et al. used 0.1 M potassium phosphate buffers between pH 4.5 and 8.0. To compare the properties of our enzyme with those of Balny et al., we investigated the hydrogen peroxide–CcP reaction in 0.1 M potassium phosphate buffers using stored enzyme. The results are shown in Figure 4. The reaction rate is essentially independent of pH between pH 4.5 and 8.0 in 0.1 M potassium phosphate buffers as reported by Balny et al. (1987).

In addition to the second-order reaction between CcP and hydrogen peroxide, a slower, hydrogen peroxide independent reaction is often observed below pH 6.0 (Loo & Erman, 1975; Balny et al., 1987). The reaction has been attributed to a minor form of the enzyme which cannot react directly with hydrogen peroxide but must isomerize to that of the native form. The amplitude of this second reaction is very small with our enzyme preparations, and we have not studied it in detail. The amplitude of the reaction increases with decreasing pH and temperature, and the rate constant is about 1–2 $s^{-1}$ at 25 °C. We have surveyed all of our old data, and the amplitude of this reaction is generally less than 2% of the total absorbance change at 424 nm for the conversion of CcP to CcP compound I at 25 °C for our stored enzyme preparations. The largest amplitude we have ever observed at 25 °C was 6% for one preparation. This hydrogen peroxide independent reaction was also observed for the fresh CcP preparations used in these studies and had an amplitude of about 1.5% that of the total absorbance change for the reaction between pH 4 and 5, 25 °C.

## Discussion

*Storage of Enzyme.* An important conclusion of this study is that storage of CcP crystals below −20 °C for periods of up to 41 months has no detectable effect on the electronic absorption spectrum or on the reaction of the native enzyme with hydrogen peroxide between pH 4 and 8. It should be noted that we purify CcP by anion-exchange chromatography in potassium phosphate buffers at pH values between 6 and 6.5. Storage of enzyme as frozen solutions, especially at acidic pH (Dasgupta et al., 1989), or of crystalline enzyme isolated by using pH 5.0 acetate buffers (Smulevich et al., 1986) appears to favor formation of aged CcP. A recent report indicates that if the enzyme, isolated with acetate buffers, is dialyzed versus 0.1 M phosphate, pH 7.0, prior to crystallization, only 5-coordinate, high-spin enzyme is obtained. No changes are observed on storage at 77 K for several months (Smulevich et al., 1989).

*pH Dependence of the Absorption Spectrum.* We have previously shown that the spectrum of CcP exhibits small pH-dependent changes in buffers containing $KNO_3$ as a supporting electrolyte (Conroy & Erman, 1978). In this report, we show that freshly isolated CcP exhibits the same spectral changes (Figures 1–3, Table II). We want to emphasize that the changes are small (Figure 1). The largest changes are on the order of 4% of the absorptivity at the Soret maximum. In order to measure the changes quantitatively, difference spectroscopy must be used (Figures 2 and 3).

When difference spectroscopy is used to monitor the spectral changes as a function of pH in 0.1 M $KNO_3$ (pH adjusted with 0.1 M $HNO_3$ or KOH), three regions are observed (Conroy & Erman, 1978). Between pH 4.8 and 6.0, good isosbestic points are observed near 398 and 422 nm. The changes that are occurring in this pH region have been attributed to the apparent ionization of an enzyme group with a $pK_a$ of 5.5 ± 0.1. The second region can be attributed to the onset of changes which eventually lead to acid denaturation below pH 4. These changes can be detected as small shifts in the isosbestic points below pH 4.8 and a decrease in absorbance at the Soret maximum by about 0.8% between pH 4.5 and 4.0. The third region occurs at alkaline pH. CcP undergoes a complex series of spectral changes above pH 7.5 (Dhaliwal & Erman, 1985; Gross & Erman, 1985). By use of difference spectroscopy, the onset of the spectral changes occurring at alkaline pH can be detected as small shifts in the isosbestic points above pH 6. Between pH 7 and 8, new isosbestic points are established at about 351, 415, and 461 nm (Conroy & Erman, 1978).

The best choice of wavelengths to determine the pH dependence of the CcP spectrum in $KNO_3$-containing buffers is 415 nm. This wavelength is an isosbestic point for the changes occurring at alkaline pH, and these processes will not contribute to the observed absorbance changes. The observed changes at 415 nm are close to the maximum changes, which occur near 410 nm in the difference spectrum (Figure 2), and the decreases in absorbance at 415 nm due to the onset of acid denaturation are minor and only detectable below pH 4.5 (Figure 3). The absorptivity changes shown in Figure 3 were fit to an equation representing the titration of a single group on the enzyme using nonlinear least-squares regression. The best-fit values for the $pK_a$ of the group are 5.4 ± 0.1 and 5.5 ± 0.1 for the fresh and stored enzyme, respectively. The change in absorptivity at 415 nm between the low-pH and

high-pH forms of the enzyme was 3.6 ± 0.2 and 2.7 ± 0.3 $mM^{-1}$ $cm^{-1}$ for fresh and stored enzyme, respectively. The solid and dashed lines shown in Figure 3 are theoretical lines calculated by using the best-fit parameters.

*Estimation of 5- and 6-Coordinate, High-Spin Forms from Absorption Data.* A key question is whether the small pH-dependent spectral changes we observe are associated with formation of a 6-coordinate, high-spin form of CcP. This is a difficult question to answer. First, within the accuracy of resonance Raman spectroscopy, our stored enzyme preparations are entirely 5-coordinate, high-spin between pH 4.5 and 6.4 in $KNO_3$-containing solutions (see Figure 4; Shelnutt et al., 1983). However, quantitation of resonance Raman spectra is difficult, and a small amount of 6-coordinate, high-spin form in a predominantly 5-coordinate sample could go undetected. Absorption measurements could be an accurate method for determining the fraction of the two coordination states in any enzyme sample if the absolute electronic absorption spectrum of the 5- and 6-coordinate, high-spin forms of ferric CcP were known.

The ratios of the absorptivity at 408/380 and 620/647 nm should be a sensitive indication of the coordination state of high-spin CcP based upon the work of Yonetani and Anni (1987) and Dasgupta et al. (1989). The data collected at pH 7.0 in Tables I and II are representative of the values obtained for native, 5-coordinate, high-spin CcP. The ratio of the 408/380-nm absorptivities averaged to 1.52 ± 0.02 for 20 observations including both freshly isolated and stored CcP in 2 different pH 7.0 buffer systems. The lowest observed value was 1.48. The ratio of the 620/647-nm absorptivities averaged to 0.74 ± 0.03 (20) with the lowest value, 0.70. These values agree quite well with those estimated from Figure 3 of Yonetani and Anni (1987) which shows the spectrum of freshly isoalted CcP in 0.1 M Mes buffer, pH 7.0.

It is more difficult to establish absorptivity ratios for 6-coordinate, high-spin CcP. Yonetani and Anni (1987) have measured the spectra of several 6-coordinate, high-spin complexes of CcP. Excluding the chloride complex, which appears atypical (Soret maximum at 413 nm and charge transfer maximum at 640 nm), the Soret maximum remains between 406 and 408 nm, and the Soret/380-nm absorptivity ratio varies between 2.22 and 2.45 for the 6-coordinate, high-spin complexes. In these same complexes, the maximum of the long-wavelength charge transfer band occurs between 616 and 620 nm, with the "620"/647-nm ratio varying between 1.85 and 3.88. Mathews and Wittenberg (1979) studied an inactive 6-coordinate form of CcP obtained in the presence of high acetate concentrations; the Soret/380-nm and 618/647-nm absorptivity ratios were 2.46 and 3.48, respectively.

Data for our high-spin CcP samples at pH 4.5 and 7.0 are collected in Table II. In phosphate buffers, at pH 4.5 and 7, the 408/380-nm and the 620/647-nm absorptivity ratios are identical, indicating that the enzyme is 5-coordinate, high-spin at both pH values. This is true for fresh and stored preparations.

In KOAc/$KNO_3$ buffer at pH 4.5, there is a small increase in the 408/380-nm absorptivity ratio and a slight decrease in the 620/647-nm absorptivity ratio. If the increase in the 408/380-nm ratio is due to the formation of the 6-coordinate form, then an increase in the 620/647-nm ratio should also occur. This is not the case, and we conclude that the increase in the 408/380-nm absorptivity ratio is not due to a change in coordination number.

Yonetani and Anni (1987) have attributed the slow hydrogen peroxide independent kinetic phase in the CcP–hydrogen peroxide reaction to the presence of 6-coordinate, high-spin enzyme. The amplitude of this reaction phase accounts for 2% or less of the total reaction for the fresh and stored samples used in this study. Consistent with this interpretation, our samples would contain a maximum of 2% 6-coordinate, high-spin CcP. We believe that the pH dependence we observe in the electronic absorption spectrum in $KNO_3$-containing buffers (Figure 3) is primarily due to a pH-dependent conformational perturbation of the 5-coordinate, high-spin state (see below).

Prior to this study, we have characterized CcP preparations by the PZ value alone. The average PZ for 82 preparations of CcP isolated in our laboratory over the last 14 years is 1.23 ± 0.05. The highest observed PZ was 1.36, and only four of the preparations had PZ values above 1.30. In view of the data presented in this study and those of Smulevich et al. (1986, 1989), Yonetani and Anni (1987), and Dasgupta et al. (1989), we believe that it is essential to further characterize CcP preparations by reporting the Soret/380-nm and 620/647-nm absorptivity ratios. We have reviewed our prior work and have calculated these absorptivity ratios for all of the preparations for which we have complete spectra. Exclusive of the six preparations whose data is presented in Table II, we have determined spectra between pH 4 and 5.5 for 17 preparations and spectra between pH 6 and 8 for 35 preparations. There is no significant difference in the ratios obtained in the two pH regions. The average Soret/380-nm absorptivity ratio is 1.56 ± 0.03 (17) between pH 4 and 5.5 and 1.54 ± 0.06 (35) between pH 6 and 8. The average 620/647-nm absorptivity ratio is 0.78 ± 0.04 (17) between pH 4 and 5.5 and 0.78 ± 0.04 (35) between pH 6 and 8. In light of the concern about the properties of 5- and 6-coordinate forms of CcP, we recommend that the Soret/380- and 620/647-nm absorptivity ratios be reported for all CcP preparations.

*Kinetic Properties.* The most dramatic observation in this study is the difference in the pH dependence of the CcP–hydrogen peroxide reaction rate between phosphate buffers and buffers which contain $KNO_3$ (Figure 4). The apparent bimolecular rate constant is influenced by an apparent $pK_a$ of 5.5 in buffers containing $KNO_3$ as supporting electrolyte (0.1 M ionic strength) for both fresh and stored enzyme. On the other hand, the rate constant is essentially independent of pH between pH 4.5 and 8 in 0.1 M potassium phosphate buffers as reported by Balny et al. (1986).

The rate decrease in $KNO_3$-containing buffers is not due to storage-related artifacts nor to the presence of 6-coordinate, high-spin CcP. More than 98% of a fresh CcP preparation reacted with hydrogen peroxide in a bimolecular process with a rate constant of 6.6 ± 1.5 $\mu M^{-1}$ $s^{-1}$ at pH 4.5 in KOAc/$KNO_3$ buffer. After storage below −20 °C for 18 months, about 97% of the same enzyme preparation reacted with a bimolecular rate constant of 6.3 ± 0.1 $\mu M^{-1}$ $s^{-1}$. The 2–3% of enzyme which reacts with the hydrogen peroxide independent rate constant of 1–2 $s^{-1}$ may represent the hexacoordinate form of CcP. It is noteworthy that there was no significant increase in the amplitude of the hydrogen peroxide independent reaction upon storage.

*Specific Ion Effects.* The dramatic difference in the kinetic properties of the CcP–hydrogen peroxide reaction in phosphate- and $KNO_3$-containing buffers must be the consequence of specific ion effects. Above pH 6, the spectral and kinetic properties of the enzyme are identical in phosphate- and $KNO_3$-containing buffers. The differences appear below pH 6 where the binding of anions would be favored. Either phosphate, nitrate, or both must bind to CcP, altering the

enzyme's properties. We know of no direct evidence concerning the binding of these two ions although there is indirect evidence that phosphate binds (Smulevich et al., 1989; Dasgupta et al., 1989).

There are basically two interpretations of the data. One, the properties of native, 5-coordinate CcP, in the absence of specific ion binding, are essentially independent of pH. Phosphate does not alter these properties whether the phosphate binds or not. However, nitrate would have to bind, altering the properties at low pH.

The second interpretation is that the properties of native, 5-coordinate CcP are pH dependent due to the apparent titration of a group on the enzyme with a $pK_a$ of 5.5 ± 0.1. The properties of the enzyme differ in the low-pH and high-pH forms. In this interpretation, nitrate is innocuous, whether it binds or not, but phosphate binding prevents the pH-dependent change. The amount of phosphate-bound enzyme must increase significantly as the concentration of phosphate increases from 10 to 100 mM since the properties of the enzyme are pH dependent in 10 mM phosphate/$KNO_3$ buffer (pH 5.5–8.0) and independent of pH in 100 mM phosphate buffer (pH 4.5–8.0) (Figure 4). There are many variations to the two interpretations presented above. Distinguishing between them will require further studies on anion binding to CcP.

*Properties of Low-pH and High-pH Forms of CcP.* Both the low-pH and high-pH forms of CcP in $KNO_3$-containing buffers are 5-coordinate, high-spin species. This conclusion is consistent with (1) the absorptivity ratios at both 408/380 and 620/647 nm (Table II), (2) the small amount, 2–3%, of the hydrogen peroxide independent phase of the CcP–hydrogen peroxide reaction at pH 4.5, and (3) the resonance Raman spectra which clearly show that CcP is 5-coordinate at both pH 4.5 and 6.4 in $KNO_3$ solutions (Shelnutt et al., 1983).

Distinct NMR spectra are obtained for the low-pH and high-pH forms of CcP (Satterlee & Erman, 1980), indicating that the two forms are in slow exchange on the NMR time scale. Both the high- and low-pH forms detected in the NMR spectra must be 5-coordinate, high-spin forms. Within experimental error, 100% of the enzyme converts between the two distinct NMR forms, while all other techniques indicate only minor amounts of 6-coordinate species.

The low-pH conformation is unreactive toward hydrogen peroxide but rapidly converts to the reactive, high-pH form on the stopped-flow time scale. To observe bimolecular kinetics at pH 4.5 and below, where more than 90% of the enzyme is in the unreactive form, the interconversion rate between the low- and high-pH forms must be greater than 1000 $s^{-1}$. The upper limit for interconversion between the two forms is 9300 $s^{-1}$, determined from the NMR properties (Satterlee & Erman, 1980). Using the upper and lower limits, the rate of interconversion is known within an order of magnitude. The rate is too slow to be due to the protonation–deprotonation of an ionizable group, suggesting that a conformational change in the protein and/or anion binding is involved in the process. Since the low- and high-pH forms of CcP react with hydrogen peroxide with such dramatically different rates, further characterization of both forms would enhance our understanding of the structural features responsible for the enzyme's reactivity with hydrogen peroxide.

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