The pH Dependence of Cytochrome $a$ Conformation in Cytochrome $c$ Oxidase*

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The pH dependence of the conformation of cytochrome $a$ in bovine cytochrome $c$ oxidase has been studied by second derivative absorption spectroscopy. At neutral pH, the second derivative spectra of the cyanide-inhibited fully reduced and mixed valence enzyme display two Soret electronic transitions, at 443 and 451 nm, associated with cytochrome $a$. As the pH is lowered these two bands collapse into a single transition at $\sim 444$ nm. pH titration of the cyanide-inhibited mixed valence enzyme suggests that the transition from the two-band to one-band spectrum obeys the Henderson Hasselbalch relationship for a single protonation event with a transition $pK_a$ of $6.6 \pm 0.1$. No pH dependence is observed for the spectra of the fully reduced unliganded or CO-inhibited enzyme. Trypsophan fluorescence spectra of the enzyme indicate that no major disruption of protein structure occurs in the pH range 5.5–8.5 used in this study. Resonance Raman spectroscopy indicates that the cytochrome $a_2$ chromophore remains in its ferric, cyanide-bound form in the mixed valence enzyme throughout the pH range used here. These data indicate that the transition observed by second derivative spectroscopy is not due simply to pH-induced protein denaturation or disruption of the cytochrome $a_2$ iron–CN bond. The pH dependence observed here is in good agreement with those observed earlier for the midpoint reduction potential of cytochrome $a$ and for the transition associated with energy transduction in the proton pumping model of Malmström (Malmström, B. G. (1990) Arch. Biochem. Biophys. 280, 233–241). These results are discussed in terms of a model for allosteric communication between cytochrome $a$ and the binuclear ligand binding center of the enzyme that is mediated by ionization of a single group within the protein.

The coupling of electron transfer reactions to the formation of transmembrane electrochemical gradients is the fundamental mechanism by which respiratory energy transduction occurs in mitochondria and aerobic bacteria (1). One of the primary sites of this coupling is cytochrome $c$ oxidase (ferrocytochrome-c:oxygen oxidoreductase, EC 1.9.3.1), a metallo-

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In this study we have made use of the high resolution power of the second derivative absorption method to evaluate the pH dependence of the cytochrome a cofactor in stable forms of the bovine enzyme. The results reported here suggest that the conformation of cytochrome a is influenced by ionization of a protein component that titrates spectrally at a pH where the enzyme is inhibited by cyanide but is independent of pH in the unliganded or CO-inhibited enzyme.

MATERIALS AND METHODS

All reagents were the highest grades commercially available, and all solutions were prepared with doubly glass-distilled water. Bovine cytochrome c oxidase was isolated from cardiac muscle as previously described and stored at −80 °C until use (11). Protein concentration was determined by the Bradford colorimetric assay using bovine serum albumin as a standard (12). Heme A concentration was determined from the spectrum of the fully reduced enzyme at 605 nm and utilizing the extinction coefficient 39.6 mM⁻¹ cm⁻¹ with respect to the enzyme (13). Enzyme activity was measured spectrophotometrically by following the loss of absorbance at 550 nm for ferrocytochrome c solutions as described by Smith (14). The purified enzyme used in these studies contained 9.9 nmol of heme A/mg of protein and displayed a maximum turnover rate of 142 s⁻¹.

The cyanide-inhibited (CN) enzyme was prepared by incubating a 300 μM solution of enzyme with 5 mM KCN at 4 °C for 48 h. The extent of cyanide binding was checked by observing the red shift of the Soret band of the enzyme from 421 to 429 nm. The CN-enzyme was then diluted to 10 μM into buffer containing either 50 mM HEPES or 50 mM MES (depending on the final pH), 100 mM KCN, and 0.1% Tween 20 (Fisher Chemical Co.). The final enzyme concentration was determined spectrophotometrically and extinction coefficient of 156 mM⁻¹ cm⁻¹ at 429 nm (13). The pH of the enzyme solution was determined at 25 °C using a Corning model 220 pH meter which was calibrated daily. For samples below pH 6.0 the CN-enzyme was first diluted into pH 7.4 buffer and then dialyzed against 100 volumes of the final pH buffer, containing 1 mM KCN, for 24 h.

The CN-inhibited mixed valence enzyme was prepared from the above solutions as follows. The CN-enzyme solution was placed in an anaerobic cuvette equipped with a stopcock and septum seal. The atmosphere above the sample was exchanged for vacuum and then nitrogen a minimum of five times. Either sodium dithionite or sodium ascorbate and N,N,N',N'-tetramethyl p-phenylene diamine were then added through the septum, and the sample was cycled through vacuum and nitrogen several more times. The sample was then incubated at 4 °C for 20 min to allow complete reduction to occur. After spectral data acquisition, the sample was removed from the cuvette, and the pH of the solution was confirmed as described above.

The fully reduced, fully reduced CN-bound, and fully reduced CO-bound enzyme forms were prepared in analogous ways using previously described procedures (9).

Optical spectra were recorded at 25 °C with a Cary 14 UV-Vis near IR spectrophotometer. The instrument was interfaced to an IBM-compatible computer which was used to control data acquisition and for digital storage of the data (OLIS, Jefferson, GA). For each experiment a buffer baseline was recorded and digitally subtracted from all subsequent spectra. Spectra of the enzymes were recorded in 1-nm steps with a spectral bandwidth of 0.5 nm. Each reported spectrum is the average of 10 such scans. The second derivatives of the spectra were obtained as previously described (9). For a single component absorption spectrum, the second derivative of absorbance with respect to wavelength displays a sharp negative extremum at the wavelength maximum of the electronic transition; this negative feature is bordered on the high and low energy sides by positive "wings." Such a pattern is observed, for example, in the second derivative spectrum of the fully reduced unliganded enzyme (9). For such a single component system, an accurate measure of the intensity of the electronic transition can be obtained by drawing a line to connect the two positive wings and then integrating the area enclosed by the roughly triangular shape described by this tangent line and the band envelope itself. We have used a similar strategy to estimate the relative intensities of the 443- and 451-nm transitions in the second derivative spectra here. Using an integration program supplied by OLIS (Jefferson, GA) we drew a line between the positive features at 435 and 446 nm and thus integrated the area under the 443-nm feature. Likewise we drew a line from 447 to 457 nm to integrate the area under the 451-nm transition. At high pH it is clear that these wavelengths correspond to the positive wings described above. It is less clear where these wings occur at lower pH, but in the interest of consistency we have chosen to use the same wavelengths for all spectra. This process is likely to underestimate the relative contribution of the 443 nm feature to the low pH spectra; however, for the type of comparative measurements we wish to make here we believe this procedure is appropriate.

Fluorescence spectra of the CN-enzyme at various pH values were obtained with a Photon Technology International alphascan spectrofluorometer (PTI, South Brunswick, NJ). Samples (1 μM en enzyme) were contained in a 1-cm quartz cuvette and excited at 280 nm. The emission was scanned from 290 to 425 nm with the emission polarizer set at 54.75°. Spectra were obtained with an integration time of 1 s/ nm and a spectral bandwidth of ~5 nm.

RESULTS

As first shown by Sherman et al. (9), the second derivative spectrum of the mixed valence CN-enzyme (i.e. a°, a°°-CN) displays two Soret transitions, associated with ferrocytochrome a, at 445 and 451 nm (9). Fig. 1 illustrates the second

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1 The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; MES, 2-morpholinoethanesulfonic acid; Tween 20, polyoxyethylene sorbitan monolaurate.
derivative spectra obtained for this form of the enzyme at three representative pH values. (Note that the spectrum of the enzyme at pH 7.4 displays a weaker contribution from the 429-nm ferricytochrome $a_3$ band than that originally reported by Sherman et al. We have determined that this discrepancy results from incomplete reduction of cytochrome $a$ in the originally reported spectrum. In terms of the ferrocytochrome $a$ transitions, however, the present spectrum agrees well with the previously reported one.) As seen in Fig. 1, there is a broadening out of the 429-nm feature of cytochrome $a_3$ as one lowers the pH from 7.4 to 5.8. This effect most likely arises from some increased heterogeneity in the ligand binding pocket of the enzyme at low pH. The most striking effect, however, is that the longer wavelength band at 451 nm is greatly diminished as the pH is lowered, so that by pH 5.8 one observes essentially a single ferrocytochrome $a$ Soret band at 443 nm.

If one fully reduces the enzyme prior to cyanide addition, one form the fully reduced CN-inhibited enzyme (i.e. $a^3$, $a_3^{3+}$-CN). The effect of pH on the second derivative spectrum of this enzyme form is similar to that for the mixed valence state, as illustrated in Fig. 2. In contrast to the case of the cyanide-inhibited enzyme forms, the second derivative spectra of the fully reduced unliganded (i.e. $a^3$, $a_3^{3+}$) and fully reduced CO-inhibited (i.e. $a^3_{CO}$, $a_3^{3+}$-CO) enzyme were insensitive to pH over the 5.5–5.8 pH range (data not shown). Likewise, the low pH-induced changes in the spectra of the cyanide-inhibited forms of the enzyme could not be mimicked by calcium ions, although this cation has been shown to mimic proton-induced changes in the difference spectra of cytochrome $c$ oxidase within mitochondria (15).

The pH-induced transition from the two-band- to single-band spectrum appears to be fully reversed when the pH is readjusted to ~7.4. To test this we prepared a solution of the fully reduced CN-inhibited enzyme in 50 mM MES, 100 mM KCl, 0.1% Tween 20, pH 5.9, as described above. The second derivative spectrum of this sample displayed a strong transition at 443 nm and a weak shoulder at 451 nm. The ratio of the integrated intensities of the 451/443-nm bands for this spectrum was 0.19. This solution was then titrated to pH 7.3 with a solution of 50 mM HEPES, 100 mM KCl, 0.1% Tween 20, pH 9.1. The second derivative spectrum was then reacquired and displayed the typical two band pattern exemplified by the spectrum in Fig. 2A. The integrated intensity ratio 451/443 nm for this spectrum was 1.13.

The single-band spectra obtained at low pH in the cyanide-inhibited enzymes are similar to that seen for the fully reduced unliganded enzyme (9). We were therefore concerned that the present results might reflect pH-induced disruption of protein structure leading to rupture of the iron–CN bond and subsequent reduction of cytochrome $a$. To address this issue we studied the intrinsic tryptophan fluorescence spectrum of the enzyme and the resonance Raman spectrum of the heme cofactors.

Fig. 3 illustrates the effects of pH on the intrinsic tryptophan fluorescence spectrum of CN-inhibited cytochrome $c$ oxidase. As first shown by Hill et al. (16) the tryptophan fluorescence maximum occurs at 328 nm in the native enzyme at neutral pH. Lowering the pH of the enzyme solution to 5.0 does not significantly affect the fluorescence wavelength maximum or quantum yield. Further lowering the pH to 2.5 leads to a significant increase in quantum yield and a red shift of the emission wavelength maximum as the protein unfolds and the otherwise buried tryptophan residues become more solvent exposed. These results suggest that over the pH range of interest, there is no major disruption of protein structure.

Fig. 4 illustrates the resonance Raman spectra from 175 to 225 and from 1325 to 1425 cm$^{-1}$ of fully reduced unliganded cytochrome $c$ oxidase at pH 7.4, and the mixed valence CN-inhibited enzyme at pH 7.4 and 5.5. In the reduced unliganded enzyme, one observes a band at 212 cm$^{-1}$ assigned to the iron-histidine stretch of five coordinate ferrocytochrome $a$. This out of plane iron-nitrogen stretch is not observed in six coordinate low spin hemes, such as the CN-bound ferricytochrome $a_3$. This band occurs at 1355 cm$^{-1}$ for the ferrous iron valence state and at 1365 cm$^{-1}$ for the six coordinate low spin ferric heme (17). Thus, if cyanide is bound to cytochrome $a_3$, one expects to observe two $v_4$, bands in the mixed valence CN-inhibited enzyme at either pH 7.4 or 5.5, indicating that cyanide remains bound to cytochrome $a_3$ at low pH. This inference is also supported by the high frequency resonance Raman data. The spectral region between 1300 and 1400 cm$^{-1}$ contains the totally symmetric stretching mode, $v_a$, of the porphyrin ring system. This band is sensitive to the oxidation state of the central iron atom of the heme; for heme $A$ the band occurs at 1355 cm$^{-1}$ for the ferrous iron valence state and at 1365 cm$^{-1}$ for the six coordinate low spin ferric heme (17). Thus, if cyanide is bound to cytochrome $a_3$ in the mixed valence enzyme one expects to observe two $v_a$ bands in the resonance Raman spectrum. As seen in Fig. 4, this expectation is met for the enzyme at both pH 7.4 and 5.5, confirming the fact that at low pH cytochrome $a_3$ remains oxidized and six coordinate. Spectra obtained in the 1500–1600 cm$^{-1}$ region of the spectrum also confirm that cytochrome $a_3$ is low spin.
in the mixed valence CN-inhibited enzyme at both pH 7.4 and 5.5 (data not shown).

Having established that the pH effect observed in the second derivative spectra is not simply due to protein denaturation, we investigated the pH dependence of the mixed valence CN-inhibited enzyme in greater detail. We have recorded the second derivative spectrum of this form of the enzyme at a number of pH values between 5.5 and 8.5 and measured the integrated areas for the 443 and 451 nm bands. Fig. 5 illustrates the effect of pH on the ratio of the integrated areas under the 451/443 nm bands. These data appear to follow the Henderson-Hasselbalch relationship for a single acid-base group and yields an estimate of the transition pK<sub>a</sub> of 6.61 ± 0.13. See "Results" for further details.

\[ y = y_{HA} + \frac{(y_{A} - y_{HA})}{10^{pK_a - pH} + 1} \]  

(1)

where \( y \) is the experimental value at any given pH, \( y_{HA} \) is the experimental value for the fully protonated form of the molecule, and \( y_{A} \) is the experimental value for the fully deprotonated form of the molecule. The data in Fig. 5 were fit to this equation using the nonlinear curve-fitting program NFIT (Island Products, Galveston, TX). The curve drawn through the data points in Fig. 5 represents the least squares best fit obtained in this way, and yields an estimate of the transition pK<sub>a</sub> of 6.61 ± 0.13 (\( x^2 = 0.0153 \)).

**DISCUSSION**

The data presented here provide clear evidence that the conformational change observed in the CN-inhibited enzyme is sensitive to proton binding at a group within the enzyme that co-localizes with a pK<sub>a</sub> of 6.6. We observe this pH dependence only in the CN-inhibited forms of the enzyme, and not in the unliganded or CO-inhibited species. Interestingly, this is exactly the behavior observed in studies of the pH dependence of the midpoint reduction potential of cytochrome a. Thus, Artzatyanov et al. (18) have shown that the reduction potential of cytochrome a is sensitive to pH in the CN-inhibited enzyme but much less so in the unliganded species (18). This group also localized the pH effect to the mitochondrial matrix side of the respiratory membrane, suggesting that the protonatable group was in closer proximity to the binuclear center (i.e. cytochrome a-CuA) than the cytochrome a cofactor. Similarly, the related terminal oxidase from Escherichia coli, cytochrome o, displays a pH dependence for the reduction potentials of its heme groups, and evidence of heme-heme interactions (19). Several groups have shown that spectral features of the CN-inhibited ferricytochrome a<sub>a</sub> site display pH sensitivity that titrate with pK<sub>a</sub> values between 6.5 and 6.9 (20, 21), while Papadopoulos et al. (22) have shown that the spectroscopic features of ferricytochrome a<sub>a</sub> titrate with an apparent pK<sub>a</sub> of 7.8 in the resting enzyme. The similarity between the pK<sub>a</sub> values obtained in these studies and that observed here suggests allosteric communication between a proton binding group in the cytochrome a-CuA pocket and cytochrome a rather than direct proton binding in the vicinity of cytochrome a.

Recently Malmström (5) has proposed a model for redox-coupled proton pumping by cytochrome c oxidase in which electrons enter cytochrome a and CuA in one conformational state of the protein, E<sub>0</sub>, but can only migrate to the binuclear oxidase center after a conformational transition to the E<sub>2</sub> state; proton translocation occurs during the E<sub>0</sub> to E<sub>2</sub> transition in this model. Malmström’s group has studied the pH dependence of cytochrome c oxidation (23) and steady state levels of cytochrome a reduction (24) for cytochrome c oxidase within proteoliposomes and has found that the transition from E<sub>0</sub> to E<sub>2</sub> conformation is associated with protonation of an acid-base group with pK<sub>a</sub> 6.4 that is protonated from the matrix side of the membrane. Taken together, these studies suggest that the differences in cytochrome a conformation observed by second derivative spectroscopy and the differences in cytochrome a reduction potential may both be manifestations of a pH-induced E<sub>0</sub> to E<sub>2</sub> like transition in the CN-inhibited enzyme.

What is the structural basis of the proposed communication between the ligand binding site of the enzyme and cytochrome a? The transition pK<sub>a</sub> values observed here and by others fall within the range 6.4–6.9, which is the expected pK<sub>a</sub> value for the side chain nitrogen of the amino acid histidine. Although cytochrome c oxidase is a multisubunit enzyme, biochemical (25) and genetic (26) data suggest that cytochrome a, cytochrome a<sub>a</sub>, and CuA are all contained within subunit I of the enzyme. It seems likely that the structural components which facilitate allostery between cytochrome a and the binuclear center would also be contained within this subunit. Hydrophathy mapping suggests that subunit I is composed of 12 membrane-spanning α helices, labeled I–XII. Comparing the
amino acid sequences of subunit I from a variety of eukaryotic and prokaryotic cytochrome c oxidases and E. coli cytochrome o one finds that there are only six highly conserved histidine residues within the transmembrane helices of subunit I (26); all six of these are expected to be directly involved in metal ligation. From a variety of molecular modeling and site directed mutagenesis studies on cytochrome c oxidase and the related cytochrome o enzyme, a consensus view of the identities of the individual metal ligands is emerging. In this view one of the conserved histidine residues of helix X (His-410) provides an axial ligand of cytochrome o, and the second conserved histidine of this helix is ligated to CuB; the remaining conserved histidines (His-91, His-273, His-322, and His-323) serve as binuclear site ligands of either cytochrome o or CuB and provide the second axial ligand of cytochrome o (27). None of these histidines would be available for acid-base chemistry while ligated to a metal center.

Thus, for a subunit I histidine to be involved in the observed pH dependence of the enzyme would require a deligation from one of the binuclear site metals to occur. It is interesting to note in this regard that the pH dependence observed here is limited to the CN-inhibited enzyme. Recent Fourier transform infrared studies indicate that cyanide, unlike carbon monoxide, binds to both cytochrome o and CuB at ambient temperatures (28). It is possible then that exogenous ligand binding at CuB might result in the displacement of a histidine ligand which would then be available for acid-base titration. However, to our knowledge there is no direct evidence for such a ligand displacement reaction at the binuclear center. Alternatively one must consider other components of the enzyme that might serve as the ionizable group. Under certain conditions other amino acid side chains, notably carboxylate residues, will display pKₐ values near 6.6 (29). There are three highly conserved aspartate residues (Asp-121, Asp-177, and Asp-401) and two conserved glutamate residues (Glu-275 and Glu-528) within subunit I; one or more of these could potentially play a role in allosteric control within the enzyme. In fact, the titratable group need not be an amino acid at all. The heme A cofactors of the enzyme contain two propionate substituents which are potential candidates for the ionizable group. While the pKₐ of propionic acid in aqueous solution is 4.87 (30), this value could easily be elevated to 6.6 by covalent attachment to the porphyrin system and by the influence of the polypeptide matrix of the enzyme.

While we cannot unambiguously define the molecular basis for allosteric at this time, it is clear that such allosteric occurs in cytochrome c oxidase. What the mechanistic significance of this allosteric communication is for the catalytic activities of cytochrome c oxidase remains an issue to be clarified by further experimental work.

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