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HEME-HEME COMUNICATION DURING THE ALKALINE INDUCED STRUCTURAL TRANSITION IN CYTOCHROME C OXIDASE

Hong Ji, Denis L. Rousseau^{*}, and Syun-Ru Yeh^{*}

Department of Physiology and Biophysics, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461

Abstract

Alkaline induced conformational changes at pH 12.0 in the oxidized as well as the reduced state of cytochrome *c* oxidase have been systematically studied with time-resolved optical absorption and resonance Raman spectroscopies. In the reduced state, the heme *a*₃ first converts from the native five-coordinate configuration to a six-coordinate bis-histidine intermediate as a result of the coordination of one of the Cu_B ligands, H290 or H291, to the heme iron. The coordination state change in the heme *a*₃ causes the alteration in the microenvironment of the formyl group of the heme *a*₃ and the disruption of the H-bond between R38 and the formyl group of the heme *a*. This structural transition, which occurs within 1 minute following the initiation of the pH jump, is followed by a slower reaction, in which Schiff base linkages are formed between the formyl groups of the two hemes and their nearby amino acid residues, presumably R38 and R302 for the heme *a* and *a*₃, respectively. In the oxidized enzyme, a similar Schiff base modification on heme *a* and *a*₃ was observed but it is triggered by the coordination of the H290 or H291 to heme *a*₃ followed by the breakage of the native proximal H378-iron and H376-iron bonds in heme *a* and *a*₃, respectively. In both oxidation states, the synchronous formation of the Schiff base linkages in heme *a* and *a*₃ relies on the structural communication between the two hemes via the H-bonding network involving R438 and R439 and the propionate groups of the two hemes as well as the helix X housing the two proximal ligands, H378 and H376, of the hemes. The heme-heme communication mechanism revealed in this work may be important in controlling the coupling of the oxygen and redox chemistry in the heme sites to proton pumping during the enzymatic turnover of CcO.

INTRODUCTION

Cytochrome *c* oxidase (CcO) is the terminal enzyme in the electron transfer chain. It catalyzes the four electron reduction of oxygen to water, thereby generating a proton motive force across the inner membrane of mitochondria in eukaryotic cells or the plasma membrane in aerobic bacteria for ATP synthesis. CcO has four redox centers, Cu_A, heme *a*, heme *a*₃ and Cu_B. Heme *a*₃ and Cu_B comprise the catalytic binuclear center (Fig. 1A), where oxygen is reduced to water; whereas Cu_A and heme *a* are electron mediators that facilitate electron transfer from the substrate, cytochrome *c*, to the binuclear center. Three possible proton pathways, named the D-, K- and H-channels based on the conserved amino acid residues near the entrance of each channel, have been identified from mutagenesis and crystallographic studies [1–5].

^{*}Corresponding Authors: Syun-Ru Yeh, Phone: (718) 430-4234; Fax: (718) 430-4230; syeh@aecom.yu.edu; Denis L. Rousseau, Phone: (718) 430-4264; Fax: (718) 430-8808; rousseau@aecom.yu.edu.

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The complete turnover of the enzyme involves an *oxidative phase* followed by a *reductive phase* [6,7]. During the *oxidative phase*, heme a_3 in the fully reduced enzyme (Compound **R**) binds oxygen to form a Fe-O₂ species (Compound **A**). The Compound **A** species receives an electron from either heme a or the Cu_B center to generate a postulated peroxo species, which is plausibly too short-lived to be detected. Upon receiving an additional electron, the O-O bond of the peroxo species is cleaved, leading to a ferryl (Fe⁴⁺=O) species (Compound **P**). The Compound **P** species is converted to Compound **F**, another ferryl species, by taking up a proton. Upon receiving another proton and electron, Compound **F** decays to the fully oxidized enzyme, Compound **O_H**.

In addition to the two protons translocated during the *oxidative phase* (one each during the **P**→**F** and the **F**→**O_H** transitions), two additional protons are translocated upon the re-reduction of **O_H** to the Compound **R** species in the *reductive phase* [6]. Intriguingly, although controversial, it has been reported that the proton translocation during the reductive phase does not take place when the enzyme is reduced from the resting **O** state instead of the **O_H** state produced immediately following the oxidative phase [8]. This observation suggests that part of the chemical energy generated in the oxidative phase must be conserved in Compound **O_H**, most likely as conformational energy, and subsequently used to drive proton translocation during the *reductive phase* [7].

Bovine heart CcO, used in this study, is an integral membrane protein. It is a homo-dimer at neutral pH [9–12]. The protein moiety of each monomer is composed of 13 different subunits with a total mass of 204 kDa [13]. The structural and functional properties of CcO have been shown to be very sensitive to pH. In intact bovine heart cells, Wilson *et al.* [14] detected a pH dependence of the E_m values of heme a and a_3 in CcO, but not the copper centers. The same phenomenon was observed in the isolated enzyme by Van Gelder *et al.* [15]. Recently, Sadoski and coworkers found that the rate for the **F**→**O_H** transition decreased by a factor of >3 as the pH was increased from 7.5 to 9.5; however, it increased by a factor of >2 if the enzyme was pre-treated with pH 10.0 buffer [16]. The accelerated **F**→**O_H** transition was attributed to the increased proton accessibility to the heme a_3 -Cu_B binuclear center due to a dimer to monomer transition, as that reported by Chan *et al.* [17] more than three decades ago. On the other hand, Riegler *et al.* reported that the steady state activity of the enzyme decreases by a factor of 65 as the pH is raised from 7.0 to 9.8 [18]. The authors suggested that under the alkaline condition the proton delivery via the K-channel becomes rate-limiting, thereby retarding the turnover of the enzyme.

In a series of time resolved studies, Einarsdottir and coworkers reported that the oxygen reaction of CcO was pH sensitive on the basis of time-resolved spectroscopic studies [19,20]. In contrast to the well-accepted sequential model (**A**→**P**→**F**→**O_H**), the authors proposed that the reaction follows two parallel pathways, in which one branch of the reaction goes through the **P** intermediate while the other branch passes through the **F** intermediate, due to the presence of two structurally distinct protein conformers such as a resting form and a more reacting pulsed enzyme. They further postulated that, due to the differences in the proton accessibilities to the binuclear center [19–21], the branching ratio between the two pathways is pH-dependent, thereby accounting for the pH sensitivity of the oxygen reaction.

The alkaline induced structural transition in CcO was first reported eighty years ago by Keilin [22], one year after its discovery. It was found that the Q band at 604 nm in the spectrum of the reduced enzyme shifted to 576 nm in potassium hydroxide treated bee muscle or yeast cells. The spectral change was proposed to be due to a conversion of the component a (now known as hemes a and a_3) into a “haemochromogen A”. A similar observation was later documented by Lemberg and co-workers [23,24] and was explained by Schiff base formation between the formyl side chains of the two hemes and the nearby amino acids bearing amine groups in the

denatured protein. To further explore this phenomenon, Babcock and coworkers employed resonance Raman, magnetic circular dichroism (MCD) and EPR spectroscopic techniques to examine the equilibrium structures of various alkaline species [25]. They reported that, in the pH range from 8.5 to 10.0, a high-spin to low-spin transition took place at heme a_3 in both oxidation states. On the other hand, at higher pH (10–12), Schiff base linkages were formed in both heme a and heme a_3 in the reduced enzyme, whereas in the oxidized derivative a μ -oxo species was produced due to dimerization of the hemes released from the denatured protein moiety.

To gain more insights into the various conformational states of bovine heart CcO in both the ferrous and ferric states, we sought to examine the time-resolved conformational changes in the enzyme following a rapid pH jump from pH 7.4 to pH 12.0. In contrast to the results of Babcock and coworkers, we found that Schiff base linkages were formed at pH 12.0 in both oxidation states. We postulate that the Schiff base formation is triggered by the coordination of a distal histidine, presumably one of the Cu_B ligands (H290 or H291), to heme a_3 .

MATERIAL AND METHODS

CcO was purified from beef hearts by the method described by Yoshikawa and coworkers [26]. The purified enzyme was dissolved in 0.01 M sodium phosphate buffer at pH 7.4 with 0.1% n-decyl- β -maltoside and stored in liquid nitrogen until further use. The CcO concentration was determined by the optical absorption difference between the fully reduced enzyme at 604 nm and the fully oxidized enzyme at 630 nm with $23.3 \text{ mM}^{-1}\text{cm}^{-1}$ as the extinction coefficient difference.

Steady-state measurements

The pH 10.5 and 12.0 CcO samples were freshly prepared by incubating 12 μM protein with an equal volume of 0.1 M pH 10.5 CAPS buffer or pH 12.0 phosphate buffer containing 0.2% n-decyl- β -maltoside at room temperature for ~5 hours prior to the measurements. For the ferrous samples, a dithionite solution was added to a N₂-purged resting CcO sample in a sealed cuvette prior to measurements.

For the optical absorption measurements, the final protein concentration was 6 μM and spectra were collected with a UV-visible (UV-Vis) spectrometer (Shimadzu UV2100U Spectrophotometer). For the corresponding resonance Raman measurements, the final protein concentration was 60 μM instead of 6 μM in order to achieve an acceptable signal to noise level. The samples were placed in a spinning Raman cell to avoid photo-damage and the integrity of the samples was confirmed before and after the Raman measurements by monitoring the optical absorption spectrum. The experimental setup for the Raman measurements is described elsewhere [27]. All the resonance Raman spectra were obtained with Soret excitation at 413.1 nm from a Kr⁺ laser (Spectra Physics Inc, Mountain View, CA). The typical laser power incident on the sample was ~8 mW.

Kinetic measurements by hand-mixing

For the pH jump experiments, the CcO solution at pH 7.4 or 10.5 was hand-mixed with equal volume of pH 12.0 sodium phosphate buffer (0.1 M, containing 0.2% n-decyl- β -maltoside) in a quartz optical cuvette with a 1 cm path length. For the ferrous samples, the protein, as well as the buffer, was purged with N₂ prior to mixing. The deadtime for the mixing was estimated to be ~1 min. The kinetic data was analyzed with a commercial software package, Matlab 6.5 (The MathWorks, Natick, MA).

Kinetic measurements by a stopped-flow system

Rapid pH jump measurements of the ferric enzyme were made with a stopped-flow system equipped with a photodiode array detector (Applied Photophysics Inc, London, UK). The mixing deadtime of the system is ~3 ms. The collected kinetics data were analyzed by a commercial global fitting routine, Pro-K, from Applied Photophysics.

RESULTS AND DISCUSSION

Resonance Raman spectra of CcO

The assignments of the heme vibrational modes of CcO are well established. The ν_4 line in the 1350 – 1380 cm^{-1} region is sensitive to the π -electron density in the porphyrin macrocycle and hence the oxidation states of the heme iron. In the ferrous form, the line is located in the 1355 – 1358 cm^{-1} region, while in the ferric form it is in the 1373 – 1375 cm^{-1} region. The ν_3 line in the 1460 – 1510 cm^{-1} region is sensitive to the spin-state and the coordination-state of the heme iron. In the ferrous form, the line is at ~1470 cm^{-1} for the five-coordinate high-spin state and it shifts to ~1500 cm^{-1} for six-coordinate low-spin complexes. In the ferric case, the line is located at ~1490, ~1475 and ~1500 cm^{-1} for five-coordinate high-spin, six-coordinate high-spin and six-coordinate low-spin complexes, respectively. The ν_2 line in the 1560 to 1590 cm^{-1} region is sensitive to the iron spin state. In both oxidation states, it is located at ~1570 and ~1585 cm^{-1} for high-spin and low-spin complexes, respectively. Although these lines are well assigned, the ν_3 line is somewhat difficult to identify in CcO due to its overlap with other modes, in the 1470 cm^{-1} region.

The formyl C=O stretching mode from heme a_3 is located at ~1664 and ~1671 cm^{-1} for the ferrous and ferric states, respectively; and the corresponding modes for heme a are located at ~1610 and 1645 – 1650 cm^{-1} , respectively. These C=O stretching modes are sensitive to both the oxidation states of the heme iron and the chemical environment surrounding the formyl group. The much lower frequency for the C=O stretching mode of heme a is due to the presence of a strong H-bond between its formyl group and a nearby amino acid residue, R38 (Fig. 1A). The high frequency of the C=O stretching mode in heme a_3 , on the other hand, is a result of the hydrophobic microenvironment surrounding the formyl group (it is noted that the formyl group in heme a_3 is sandwiched by H290 and A359).

Alkaline transition in the ferrous derivative

The optical absorption spectra of ferrous CcO at pH 7.4, 10.5 and 12.0 are shown in Fig 2A. The corresponding high frequency region of the resonance Raman spectra are shown in Fig 2B. In the native state (**N_R**) at pH 7.4, the Soret and Q bands are located at 443 and 604 nm, respectively. The 1472 (ν_3), 1570 (ν_2) and 1664 (formyl) cm^{-1} lines in the resonance Raman spectrum are assigned to the five-coordinate high-spin heme a_3 with a single axial histidine ligand (H376 as shown in Fig. 1). On the other hand, the 1585 (ν_2) and 1612 (formyl) cm^{-1} lines are attributed to the six-coordinate low-spin heme a with two axial histidine ligands (H61 and H378). The shoulder at 1624 cm^{-1} is assigned to the C=C stretching mode of the vinyl groups from both hemes.

When the pH is raised to 10.5, only minor changes are detected in the optical absorption and the resonance Raman spectra. In contrast, when the pH is further raised to 12.0, the Soret band shifts from 443 to 428 nm and the visible Q band shifts from 604 to 575 nm, with a concomitant development of a shoulder at 594 nm (Fig. 2A). The changes in the optical absorption spectrum are accompanied by dramatic changes in the resonance Raman spectrum (Fig. 2B), in which new broad lines at 1585 and 1629 cm^{-1} appear at the expense of the lines found at neutral pH conditions described above.

Structure of the S_R species

Both the optical absorption and Raman spectra of the fully reduced species at pH 12.0 (referred to as S_R hereafter) are nearly identical to those of the six-coordinate low-spin ferrous heme model complex with two nitrogenous ligands coordinated to the heme iron and a Schiff base linkage formed on the formyl group as shown in Figs. S1 and S2 in the Supplemental Material and listed in Table I [28]. Based on the similarity to the spectra of the model complex, we postulate that, in the S_R state, the formyl groups of hemes a and a_3 form Schiff base linkages with nearby amino acid residues bearing a primary amine sidechain group via the following reaction:

This assignment is consistent with the appearance of the C=N stretching mode at 1629 cm^{-1} and the concurrent disappearance of the two formyl C=O stretching modes at 1612 and 1664 cm^{-1} in the Raman spectrum of the S_R species (Fig. 2B).

Two amino acids, lysine and arginine, which contain primary amine sidechain groups, may form a Schiff base linkage with the heme formyl group through a nucleophilic addition reaction. Although lysine is a better nucleophile than arginine, all the lysine residues in CcO are located more than 20 Å away from the heme formyl groups. On the other hand, R38 is only 2.9 Å away from the formyl group of the heme a , and it forms an H-bond with it in the native state (Fig. 1A). Hence, it is the most likely residue to react with the formyl group of heme a . We postulate that the alkaline-induced structural transition disrupts the H-bond between R38 and the formyl group of heme a , thereby allowing the formation of the Schiff base linkage between them. For heme a_3 , the closest ligand that may form a Schiff base linkage with the formyl group is R302, which is 13 Å away from it (Fig. 1A). Although this distance is too long for the nucleophilic addition reaction to take place directly, the rupture of the $\text{Cu}_B\text{-H290}$ or $\text{Cu}_B\text{-H291}$ bond and the subsequent coordination of the H290 (or H291) to the heme a_3 iron associated with the alkaline transition (*vide infra*) may trigger the movement of the peptide fragment linking R302 and H290/H291 (Fig. 2A) towards the heme a_3 , thereby bringing the amine sidechain group of R302 to close proximity to the formyl group to form the Schiff base.

The Raman data shown in Fig. 2B suggests that both the heme a and a_3 in the S_R state are coordinated by two histidine ligands, indicating that heme a retains its native axial coordination whereas heme a_3 is coordinated by not only the original proximal ligand, H376, but also an additional non-native histidine ligand. Four histidine residues, H240, H290, H291 and H368, are found in the vicinity of heme a_3 , as highlighted in Fig. 1A. Among the four histidine residues, H368 is 11.0 Å away from the distal binding site of heme a_3 and forms an H-bond with one of the propionate groups of the heme. Thus, it is unlikely to be the non-native heme a_3 ligand. H240, one of the three histidine residues coordinated to the Cu_B atom, forms a covalent linkage with Y244 as a result of a post translational modification (Fig. 1A). Due to the steric constraints imposed by the post translational modification, H240 is also an unlikely axial ligand for heme a_3 . Consequently, we postulate that the non-native heme a_3 ligand is either H290 or H291, the other two histidine residues that are coordinated to Cu_B in the native structure. The coordination of the Cu_B ligands to heme a_3 is not unprecedented. In *Rhodobacter sphaeroides* CcO, the mutation of Y288 (equivalent position to Y244 in the bovine enzyme) to Phe results in the release of the Cu_B and the concomitant coordination of one of its His ligands to the heme iron [29] to form a six-coordinate low-spin heme a_3 .

To confirm that at least one of the two axial ligands of heme a_3 in the S_R state is a histidine, we treated S_R with CO. The instant formation of the CO-bound complex indicates that one of the two axial histidine ligands, possibly the new ligand (i.e. H290 or H291) can be easily

displaced by CO. The Fe-CO stretching ($\nu_{\text{Fe-CO}}$) and C=O stretching ($\nu_{\text{C=O}}$) modes of the CO-derivative were identified at 499 and 1968 cm^{-1} , respectively, in the resonance Raman spectrum (data not shown). The data lie on the well-known $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C=O}}$ inverse correlation line for proteins containing a proximal histidine/imidazole ligand [30], confirming that at least one of the two heme a_3 axial ligands in the S_R state is a histidine. The relatively low $\nu_{\text{Fe-CO}}$ frequency indicates that the heme-bound CO is in an open pocket, i.e. there is no direct electrostatic interaction between the CO and the protein matrix, suggesting that the dissociated histidine ligand is not in the vicinity of the heme-bound CO.

Kinetics of the Schiff base formation in ferrous CcO

To examine the Schiff base formation kinetics, the CcO sample at pH 7.4 was mixed with excess buffer at pH 12.0 in a sealed quartz cuvette. The reaction was followed by optical absorption and resonance Raman spectroscopies. Optical absorption data show that the reaction follows a two-step mechanism. Within the mixing deadtime (~ 1 min), the Soret band shifts from 443 to 436 nm ($k > 2 \times 10^{-2}$); it is followed by a slower transition from 436 to 428 nm as shown in Fig. 3A. The presence of clear isobestic points during the slow phase indicates a one-to-one transition from the 436 nm intermediate to the 428 nm Schiff base product (S_R) as illustrated in eq. 2:

Here N_R and I_R represent the native enzyme and the 436 nm intermediate, respectively. To determine k_2 , the time-resolved spectra were deconvoluted into a linear combination of the spectra of I_R (436 nm) and S_R (428 nm) by a previously described procedure [31]. Good fits with small residuals confirm that no other intermediates are populated during the reaction. The resulting population of I_R is plotted as a function of time in the inset of Fig. 3A. The associated kinetic trace can be fitted with a single exponential function with a rate constant of $\sim 4 \times 10^{-4} \text{ s}^{-1}$ as listed in Table II.

Consistent with the optical absorption data, the time-resolved resonance Raman spectra show a rapid conversion of the native N_R species to the I_R intermediate within the mixing deadtime (~ 1 min), which is followed by a slower transition from I_R to S_R (Fig. 3B). Like the optical absorption data, each time-resolved spectrum in this figure can be deconvoluted into a linear combination of I_R and S_R with negligible residuals. The resulting time evolution of S_R can be fitted with a single exponential function with a rate constant of $\sim 4 \times 10^{-4} \text{ s}^{-1}$ (see the inset in Fig. 3B), consistent with the optical absorption data shown in Fig. 3A.

Similar time-resolved optical absorption and resonance Raman spectra were obtained following the pH jump from 10.5 to 12.0 (see Fig. S3 in the Supplement Material). The kinetic traces obtained by spectral deconvolution of the data exhibit a rate constant of $\sim 4 \times 10^{-4} \text{ s}^{-1}$, which is identical to that observed for the pH 7.4 $\rightarrow$ 12.0 jump reaction, confirming that the properties of the ferrous protein are similar in the pH range from 7.4 to 10.5.

Structural properties of the I_R intermediate

The I_R intermediate, which is formed within one minute following the pH jump, is characterized by the optical absorption transitions at 436 and 594 nm and vibrational modes at 1357, 1585 and 1634 cm^{-1} . The optical absorption spectrum is very similar to that of the six-coordinate imidazole bound ferrous heme a model compound in CH_2Cl_2 [28] as shown in Fig. S1 in the Supplementary Material and Table I, suggesting that heme a and heme a_3 in the I_R intermediate are both coordinated by two histidine ligands and they are in *hydrophobic* environments.

The data suggest that the ligand coordination states of heme *a* and heme *a*₃ in **I_R** are the same as those in the **S_R** state, i.e. heme *a* is coordinated by H61 and H378, whereas heme *a*₃ is coordinated by H376 and H290 (or H291). Although the optical absorption spectra indicate that both heme groups are in hydrophobic environments, the formyl C=O stretching frequency at 1634 cm⁻¹ is essentially the same as that reported for heme *a* model complex in an *aqueous* environment, which is 10 cm⁻¹ lower than that in CH₂Cl₂ (see Fig. S4 in the Supplementary Material and Table I). The formyl group of heme *a*₃ in the native protein is sandwiched by H290 and A359; thus it is in a rather hydrophobic environment. The shift of the formyl C=O stretching frequency of heme *a*₃ from 1664 to 1634 cm⁻¹ thus suggests that the environment of the formyl group is altered from a hydrophobic to an aqueous environment, plausibly due to conformational change induced by the breakage of the Cu_B-H290 or Cu_B-H291 bond and the subsequent coordination the H290 or H291 to the heme iron. On the other hand, the formyl group of heme *a* in the native state forms an H-bond with R38 (Fig. 1A). The shift of the formyl C=O stretching frequency of heme *a* from 1610 to 1634 cm⁻¹ suggests that this H-bond is absent and the formyl group is exposed to an aqueous environment.

In contrast to the formyl group, the vinyl C=C stretching mode is not evident in the spectrum of the **I_R** intermediate. The vinyl line at 1625 cm⁻¹ is noticeably strong in the spectrum of heme *a* model in an aqueous environment, but it is very weak in CH₂Cl₂ as shown in Fig. S4 in the Supplementary Material [28]. The data hence suggest a hydrophobic environment for the vinyl groups of hemes *a* and *a*₃ in the **I_R** intermediate. Taken together we conclude that both heme *a* and heme *a*₃ in the **I_R** intermediate have six-coordinate bis-histidine coordination and are in general in *hydrophobic* surroundings, but with the formyl groups exposed to an aqueous environment.

Mechanism for the alkaline-induced structural transition in the ferrous derivative

As discussed above, the formation of the Schiff base linkages involves a nucleophilic addition of R38 and R302 to the formyl group of heme *a* and *a*₃, respectively. The single exponential kinetic behavior of the **I_R**→**S_R** transition indicates that the formation of the two Schiff base linkages are rate-limited by the same processes. We postulate that the conformational change, required for bringing the arginine residues in close proximity to both heme formyl groups, is the rate-limiting step for the reaction, and the conformational change is triggered by the coordination of the sixth histidine ligand (H290 or H291) to the heme *a*₃ iron. We propose that the histidine coordination reaction triggers the movement of the polypeptide fragment holding R302 towards the heme *a*₃ formyl group, thereby allowing the formation of the Schiff base linkage between the two groups. Concurrently, the conformational change in the heme *a*₃ site is transmitted to the heme *a* region, possibly via the H-bonding network involving R438, R439 and the propionate groups of the two hemes as well as the helix X housing the two proximal heme ligands, H378 and H376 (Fig. 1), thereby triggering the formation of the second Schiff base linkage between the R38 side chain and the heme *a* formyl group. It should be noted that the homodimeric CcO enzyme is expected to separated into monomers at pH 12, which may facilitate the conformational change linked to the alkaline transition.

Alkaline transition in the ferric CcO

The optical absorption and resonance Raman spectra of the ferric protein at pH 7.4, 10.2, 10.5 and 12.0 are shown in Fig. 4. In the native state (**N_O**) at pH 7.4, the Soret and Q-band optical transitions are at 420 and 599 nm, respectively. The heme *a* is coordinated by H61 and H378 with a six-coordinate low-spin configuration as indicated by the Raman lines at 1501 and 1586 cm⁻¹ for the ν_3 and ν_2 modes, respectively. The heme *a*₃, on the other hand, is coordinated by H376 and possibly a water molecule or a hydroxide ion, with a predominantly six-coordinate high-spin configuration as reflected by the ν_4 and ν_2 lines at 1473 and 1573 cm⁻¹, respectively.

The formyl C=O stretching modes for heme *a* and heme *a*₃ are identified at ~1647 and ~1671 cm⁻¹, respectively.

When the pH is increased to 10.2, the Soret band shifts from 421 to 424 nm (Fig. 4A), while the Q band remains almost the same. A further increase in pH to 10.5 causes the Soret to further shift to 427 nm. The red-shifts in the Soret band are associated with the enhancement of the low-spin Raman marker line at 1586 cm⁻¹ at the expense of the high-spin marker line at 1573 cm⁻¹ (Fig. 4B). The data indicate that at pH 10.2–10.5 heme *a*₃ converts from a predominantly high-spin to a low-spin state (referred to as **L_O** hereafter), plausibly due to coordination of a hydroxide ligand in a low-spin configuration. The Raman data also show that the microenvironment of the formyl groups of both heme *a* and heme *a*₃ remains unchanged and there is no Schiff base formation under these alkaline conditions, since no modifications appear in the formyl C=O stretching modes at 1647 and 1672 cm⁻¹.

When the pH is further raised to 12.0, the Soret band shifts to 407 nm, and the Q-band is replaced by a new band at 633 nm (Fig. 4A), indicating that the oxidized enzyme at pH 12.0 (referred as **S_O** hereafter) has five-coordinate high-spin hemes [32]. Consistent with this conclusion, in the Raman spectrum, a new strong line appears at 1490 cm⁻¹ (Fig. 4B), characteristic of five-coordinate high-spin hemes. The relative intensities of the 1373 (ν₄), 1490, 1579 and 1634 cm⁻¹ lines are very similar to those found in several five-coordinate heme proteins, such as the *Scapharca inaequivalvis* hemoglobin, the H20A mutant of bacterial heme oxygenase HmuO, the H93G mutant of sperm whale myoglobin and nitric oxide synthase [33–38]. It is important to note that the unique spectral features of these five-coordinate heme proteins (especially the relatively strong intensities the 1490 and 1579 cm⁻¹ lines with respect to the 1373 cm⁻¹ line) do not seem to be sensitive to the identity of the sole axial heme iron ligand, which can be hydroxide, histidine or thiolate; we postulate that they are spectral markers for a five-coordinate heme with the porphyrin macrocycle that has an out-of-plane distortion [27]. On the other hand, the two strong Raman modes at 1579 and 1634 cm⁻¹ are consistent with those of Schiff base derivatives as shown in Fig. S5 in the Supplemental Material [28]. Most importantly, the 1634 cm⁻¹ line is nearly the same as that reported for the C=N stretching mode of the Schiff base derivative of free heme *a*.

Taken together, the data indicate that the formyl groups of both heme *a* and *a*₃ in the **S_O** species form Schiff base linkages with nearby amino acids, possibly R38 and R302 as proposed for the **S_R** state of the reduced enzyme, although, in contrast to the six-coordinate low-spin configuration found in the **S_R** state, both hemes in the **S_O** state exhibit a five-coordinate high-spin configuration, with their porphyrin macrocycle twisted out of plane.

Kinetics of the Schiff base formation in the ferric CcO

To examine the kinetics of the Schiff base formation in the ferric enzyme, the native protein at pH 7.4 was mixed with excess pH 12.0 buffer in an optical cuvette. The time-resolved structural changes were monitored with optical absorption and resonance Raman spectroscopies. The optical absorption data show that, immediately following the pH jump (t ~1 min), the Soret band shifts from 420 to 419 nm and the Q band shifts from 599 to 605 nm. Subsequently, the Soret band further shifts to 407 nm, culminating in the **S_O** state (Fig. 5B). When the first spectrum (~1 min) is ignored, the time-dependent spectra exhibit excellent isosbestic points, indicating a two-state transition and a missing kinetic phase. To further examine the missing phase, the same reaction was initiated and followed by a stopped-flow system with a dead-time of 3 ms. As shown in Fig. 5A, the stopped-flow data show a gradual decrease in the Soret intensity, followed by a blue-shift in the Soret maximum from 420 to 411 nm. These spectral changes are associated with the diminishment of the 600 nm band and the concurrent appearance of a new band at 635 nm.

The stopped-flow data can be fitted with a three-state model, $\text{N}_\text{O} \rightarrow \text{N}_\text{O}' \rightarrow \text{I}_\text{O}$, with Soret maxima of three species at 420, 420 and 411 nm, respectively (Fig. 6). The rate constants were determined to be 0.6 and 0.05 s^{-1} , as listed in Table II. Combining the kinetic data shown in Fig. 5A and B, the pH jump reaction is best described by the following mechanism.

As shown in Fig. 6, the spectral properties of N_O' are very similar to those of N_O , suggesting that both heme *a* and heme *a*₃ are in native-like spin and coordination states. The small spectral differences between N_O' and N_O are plausibly a result of small conformational changes to the protein matrix surrounding the heme groups. The absorption spectrum of the I_O state, on the other hand shows a five-coordinate high-spin character, as indicated by the presence of the broad charge transfer band at 635 nm.

To determine the rate constant for the $\text{I}_\text{O} \rightarrow \text{S}_\text{O}$ transition, each spectrum shown in Fig. 5B, except the first time point, was deconvoluted into a linear combination of the second and last spectra. The time-dependent change in the population can be fitted with a bi-exponential model with rate constants of 7×10^{-3} and $1 \times 10^{-3} \text{ s}^{-1}$ (Table II), as shown in the inset of Fig. 5B. The bi-exponential kinetics, instead of a single exponential kinetics as that found in the ferrous enzyme (Fig. 3A), suggests that the Schiff-base formation in heme *a* and *a*₃ may not be synchronous. To further examine the $\text{I}_\text{O} \rightarrow \text{S}_\text{O}$ transition, the same pH jump experiment was followed by resonance Raman spectroscopy. As shown in Figs. 7A, the intensities of the two formyl C=O stretching modes at 1636 and 1670 cm^{-1} gradually convert to a single mode at 1634 cm^{-1} , reflecting the reaction of the formyl groups with nearby amino acids (plausibly R38 and R302 as discussed above) to form the Schiff base linkages. The spectral changes are accompanied by (1) an increase in the intensity of the 1490 cm^{-1} mode at the expense of the 1470 cm^{-1} mode, indicating a transition from a six-coordinate high-spin to a five-coordinate high-spin species, and (2) an increase in the intensity ratio of the 1490 and 1373 cm^{-1} modes, possibly reflecting the out-of-plane distortion of the heme groups. Consistent with the optical absorption data, these Raman modes exhibit nonsynchronous temporal changes. As shown in Fig. 7B, the decay rate of the 1490 cm^{-1} mode ($3.3 \times 10^{-3} \text{ s}^{-1}$) is similar to that of the 1670 cm^{-1} mode ($4.9 \times 10^{-3} \text{ s}^{-1}$), but is significantly faster than that of the 1373 cm^{-1} line ($7.8 \times 10^{-4} \text{ s}^{-1}$), indicating that the spin-transition, Schiff base formation and heme distortion associated with the $\text{I}_\text{O} \rightarrow \text{S}_\text{O}$ transition are not cooperative processes.

To further examine how the spin and coordination state of heme *a*₃ affects the Schiff base formation, we examined the pH jump reaction from 10.2 to 12.0. As shown in Fig. 8A, immediately following the pH jump from 10.2 to 12.0 ($t \sim 1 \text{ min}$), the Soret transition shifts from 424 to 412 nm, with only a shoulder remaining at 424 nm. As the reaction progresses, the Soret band further shifts to 407 nm; in addition, at the expense of the 601 nm band, a new band appears at 633 nm, consistent with the formation of the S_O species. The spectral change is similar to the trend observed during the pH 7.4 to 12.0 jump (Fig. 5B), suggesting that the $\text{L}_\text{O} \rightarrow \text{S}_\text{O}$ reaction also goes through the I_O intermediate. To evaluate this hypothesis, the early missing kinetic phase was evaluated with a stopped-flow system (Fig. 9). Global fitting analysis of the data is consistent with the presence of two species, L_O and I_O , which have Soret maxima at 424 and 411 nm, respectively (Fig. 9B). The spectral features of the I_O intermediate obtained from the fitting is almost identical to that observed during the pH 7.4 to 12.0 jump reaction, confirming that the $\text{L}_\text{O} \rightarrow \text{S}_\text{O}$ reaction, like the $\text{N}_\text{O} \rightarrow \text{S}_\text{O}$ reaction, goes through the I_O intermediate as illustrated below.

As shown in the inset in Fig. 8A, the $\text{I}_\text{O} \rightarrow \text{S}_\text{O}$ transition can be fitted with a bi-exponential function with rate constants of 3×10^{-3} and $0.3 \times 10^{-3} \text{ s}^{-1}$, which are similar to those obtained for the pH 7.4 to 12.0 jump reaction (7×10^{-3} and $1 \times 10^{-3} \text{ s}^{-1}$). On the other hand, the rate constant for the $\text{L}_\text{O} \rightarrow \text{I}_\text{O}$ transition is estimated to be 0.1 s^{-1} (Fig. 9A), which is also similar to that for the $\text{N}_\text{O}' \rightarrow \text{I}_\text{O}$ transition in the pH 7.4 to 12.0 jump reaction (0.05 s^{-1}).

The resonance Raman data associated with the $\text{I}_\text{O} \rightarrow \text{S}_\text{O}$ transition in the pH 10.2 to 12.0 jump (Fig. 8B) show similar time-dependent spectral changes as compared to those observed during the pH 7.4 to 12.0 jump reaction (Fig. 7A), confirming that I_O is a common intermediate for the two pH jump reactions and, indeed, is an obligatory intermediate for the Schiff base formation. Additional kinetic studies show that it takes $\sim 200 \text{ min}$ for the N_O state to convert to the L_O state (Fig. 10), much longer than the lifetime for the $\text{N}_\text{O} \rightarrow \text{S}_\text{O}$ transition, indicating that the $\text{N}_\text{O} \rightarrow \text{S}_\text{O}$ reaction does not go through the L_O intermediate.

Structural properties of the I_O intermediate

The I_O intermediate is characterized by optical absorption transitions at 411 and 635 nm, suggesting that both heme *a* and heme *a*₃ are five-coordinate high-spin, possibly with histidine as the sole axial ligands. To evaluate further the structural properties of the 411 nm intermediate, the time-resolved resonance Raman spectra obtained during the pH jump experiments as shown in Figs. 7A and 8B were analyzed. In the first two spectra in Fig. 7A, associated with the pH 7.4 to 12.0 jump there are weak lines at ~ 1473 and 1670 cm^{-1} , which we attribute to a small residual of the N_O' form of heme *a*₃ and which decays rapidly. Similarly, in Fig. 8B, for the pH 10.2 to 12.0 jump, the first spectrum contains a small contribution from the L_O species. It was found that the subsequent Raman spectra are very similar to each other in both measurements and they are analogous to those of heme proteins with a five-coordinate high-spin configuration with a histidine as the single axial ligand. Consequently, we assign that the I_O intermediate as primarily a five-coordinate high-spin species with a single histidine bound to each of the two heme iron atoms. On the other hand, as shown in Fig. 7A, the broad band in the $1650 - 1670 \text{ cm}^{-1}$ region, replacing the formyl C=O stretching modes of the native enzyme at 1647 and 1671 cm^{-1} and the broad band at $\sim 1636 \text{ cm}^{-1}$, are very similar to those of the ferric heme *a* model in an aqueous environment as shown in Fig. S6 in the Supplemental Material [28], indicating that both hemes *a* and *a*₃ are in a hydrophilic environments.

Structural basis for the alkaline-induced transition in the ferric derivative

On the basis of the data discussed above, we postulate that pH jump from 7.4 to 12.0 first induces conformational changes to the protein matrix (the $\text{N}_\text{O} \rightarrow \text{N}_\text{O}'$ transition). In the following $\text{N}_\text{O}' \rightarrow \text{I}_\text{O}$ transition, the Cu_B -H290 or Cu_B -H291 bond is broken. It enables the subsequent coordination of the H290 or H291 to the heme *a*₃. In contrast to the ferrous enzyme, in which the H290 or H291 coordination leads to the formation of the metastable six-coordinate low-spin I_R intermediate, the same coordination reaction in the ferric enzyme plausibly triggers the rupture of the native proximal iron-H376 bond in heme *a*₃ and the plausibly the iron-H378 bond in heme *a* (see the structure in Fig. 1B), which results in the five-coordinate high-spin heme *a* and heme *a*₃ and the exposure of the formyl groups to an aqueous environment. The conformational changes underlying the $\text{N}_\text{O}' \rightarrow \text{I}_\text{O}$ transition is most likely mediated by movement of the helix X housing H376 and H378 (Fig. 1B), as well as the H-bonding network involving R438, R439 and the propionate groups of the two hemes (Fig. 1A) as discussed in the ferrous case. During the subsequent $\text{I}_\text{O} \rightarrow \text{S}_\text{O}$ transition, an additional conformational changes occurs, similar to that proposed for the ferrous enzyme, allowing the R38 and R302

residues to move to the neighborhood of the formyl groups to form the Schiff base linkages and the out-of-plane distortion of the hemes. The kinetics of the individual Raman modes indicate that the decay of the **I_O** species involves the formation of the Schiff base first, as evidenced by the changes in the intensity of the 1670 and 1490 cm⁻¹ modes with similar rate constants 3.3×10^{-3} and 4.9×10^{-3} s⁻¹, and, subsequently, by changes in the distortion of the heme macrocycle, as shown by the loss of intensity of the mode at 1373 cm⁻¹ at a rate of 7.8×10^{-4} s⁻¹.

Reduction kinetics of the Schiff base modified CcO

It is important to note that although bis-histidine coordinated species was postulated as an obligatory intermediate during the **N_O'**→**I_O** transition, it was not observed during the pH jump experiment. To test if the postulated bis-histidine intermediate can be populated during the reduction reaction of **S_O** with five-coordinate high-spin hemes to **S_R** with bis-histidine six-coordinate low-spin configuration, we rapidly mixed the **S_O** species with dithionite under anaerobic condition and followed the reaction by optical absorption and resonance Raman spectroscopies. We found that, within the 1 min mixing deadtime, the Soret band shifted from 407 to 427 nm with only a small residual shoulder at 407 nm (Fig. 11A). It was followed by a progressive change to the 428 nm species, which exhibited optical and Raman spectra (Fig. 11B) identical to those of **S_R**, confirming the reduction of **S_O** to **S_R**. The time-resolved optical absorption spectra can be deconvoluted into a linear combination of **S_O** and **S_R**. Similarly, in the resonance Raman spectra, the first spectrum detected after mixing is a spectrum very similar to that of the reduced pH 12.0 spectrum that reaches completion within several minutes. The absence of the bis-histidine coordinated ferric intermediate during the reaction indicates that it is not stable enough to accumulate to a detectable level even if it is an obligatory intermediate for the reduction reaction.

On the basis of a similar pH dependent study, Babcock and coworkers reported that, instead of the Schiff base product (**S_O**), the pH jump of the ferric enzyme to 12.0 resulted in a μ-oxo dimer. For the μ-oxo dimer to form, either the two hemes have to be completely released out of the protein matrix or they have to move close enough to each other within the protein matrix to be bridged via an oxygen atom. Considering the fact that the heme groups are tightly anchored to the protein moiety by the long hydrophobic farnesyl side chains and that they are separated by the helix X in the native matrix, for either scenario to take place a large-scale conformational changes have to take place. In contrast, our results demonstrate that the reduction of **S_O** produces **S_R**, with heme *a* and *a*₃ in six-coordinate bis-histidine coordination states, thereby mitigating against the possibility that **S_O** is a μ-oxo dimer, otherwise a reduced form of the μ-oxo dimer would be expected.

Conclusions

We have demonstrated that following the pH jump from 7.4 to 12.0, the heme *a*₃ of the reduced CcO first converts to a six-coordinate bis-histidine configuration, as a result of the breakage of the H290-Cu_B or H291-Cu_B bond and the consequent coordination of H290 or H291 to the heme iron. The coordination of H290 or H291, to heme *a*₃ triggers a series of conformational changes, culminating in the formation of Schiff base linkages between the formyl groups of heme *a* and *a*₃ and nearby amino acid residues, plausibly R38 and R302, respectively. In the ferric enzyme, a similar Schiff base formation was observed following the pH jump. Like the ferrous enzyme, the Schiff base formation is triggered by the coordination of H290 or H291, to heme *a*₃. Unlike the ferrous enzyme, in which the hemes remain in a six-coordinate bis-histidine form, it was followed by the breakage of the native proximal H378-iron and H376-iron bonds in heme *a* and *a*₃, respectively, leading to five coordinate hemes. Nonetheless, the synchronous formation of the Schiff base linkages in heme *a* and *a*₃ in both oxidation states

relies on the structural communication between the two hemes via the H-bonding network involving R438 and R439 and the propionate groups of the two hemes as well as the helix X housing the two proximal ligands, H378 and H376, of the hemes (Fig. 1). The heme-heme communication mechanism revealed in this work may be important in controlling the coupling of the oxygen and redox chemistry in the heme sites to proton pumping during the enzymatic turnover of CcO.

This type of heme *a*-heme *a*₃ communication mechanism is not unprecedented. In the *aa*₃-type CcO from *Rhodobacter sphaeroides* (CcO_{RS}), the mutation of R52 (identical to R38 in the bovine enzyme) to alanine results in a completely inactive enzyme [39]. The formyl C=O stretching mode of heme *a* in this mutant shifts to higher frequency due to the absence of the H-bond donated from R52; in addition, the perturbation in the formyl group of heme *a* causes the heme *a*₃ to convert to a six-coordinate low-spin form, possibly a bis-histidine species. In the bovine CcO, the R38 (or R52 in CcO_{RS}) in fact has been proposed as part of a proton pumping H channel [5] and the R438 and R439 were demonstrated to be part of the proton exit channel [40]. In the *aa*₃-type quinol oxidase from a hyperthermophilic archaeon, *Acidianus ambivalens*, reduction induced conformational changes to the heme *a*₃ formyl group, which were proposed to be important for the redox-coupled proton-translocation mechanism of this enzyme [35]. In the cytochrome *bo*₃ quinol oxidase from *Escherichia coli*, reduction of the enzyme induced loss of one of the three Cu_B histidine ligands (either H290 or H291 based on the bovine enzyme numbering), suggesting that during the catalytic cycle of the enzyme the cleavage of the H290-Cu_B or H291-Cu_B bond may be an integral part of the proton pumping mechanism [41]. A sensitive coupling mechanism between the two hemes, as suggested by the data reported here, appears to be pervasive in members of the oxidase terminal enzyme family.

The stable Schiff base products (**S_O** and **S_R**) of CcO in both oxidation states indicates that the protein matrix near the newly formed Schiff bases must retain an environment with limited accessibility to solvent water molecules, otherwise they would be readily hydrolyzed. Alkaline induced conformational changes in CcO have been reported to be coupled to the dimer-monomer transition [17]. Recent data showed that the F→O transition during the oxygen reduction chemistry is accelerated in the monomeric form, which was attributed to a more open structure in the monomer (hence better proton accessibility to the heme-copper binuclear center) [16]. Here we showed that the Schiff base formation kinetics of the ferrous protein following the pH 7.4→12.0 jump is indistinguishable from that of the pH 10.2→12.0 jump reaction, indicating that the establishment of the Schiff base linkage in the dimeric protein is as efficient as that in the monomeric protein, and that Schiff base formation in the ferrous enzyme is not rate-limited by the dissociation of dimer into monomers. Similarly, in the ferric case, the Schiff base formation kinetics are not significantly affected by the initial pH conditions applied, again indicating that the reaction is not rate-limited by the monomerization process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. The local structure of heme *a* and heme *a*₃-Cu_B binuclear active center of the fully oxidized CcO enzyme (PDB code: 1V54)

In the catalytic reaction oxygen binds to the distal side of heme *a*₃, which is coordinated by H376 on the proximal side of the heme macrocycle. Cu_B is ~5 Å from the heme iron atom and is coordinated by three histidine residues, one of which, H240, forms a post-translational linkage to Y244. The histidine and arginine residues in the vicinity of the heme *a* and heme *a*₃ are shown to highlight their interactions with the hemes. The dotted lines indicate the H-bonds between these residues and the hemes.

Fig. 2. The pH-dependent optical absorption and resonance Raman spectra of ferrous CcO
 The optical absorption spectra are shown in panel (A) and the resonance Raman spectra are shown in panel (B). The excitation wavelength for the resonance Raman measurements was 413.1 nm from a Kr^+ laser.

Fig. 3. The time-dependent optical and resonance Raman spectra obtained following the pH jump from pH 7.4 to 12.0

The reaction was initiated by hand-mixing with a deadtime of ~ 1 min. The spectra listed in (A) were acquired at 1, 4, 7, 13, 18, 27, 39, 71 and 196 minutes following the initiation of the reaction. The spectra listed in (B) were acquired at 1, 4, 18 and 180 minutes following the initiation of the reaction. The arrows indicate the trends for the changes in the optical or Raman intensity as the reaction progresses. The inset shows the kinetic trace obtained by spectral deconvolution of the time-dependent data associated with the $I_R \rightarrow S_R$ transition as described in the text. The smooth grey lines are single exponential fits to the data with a rate constant of $4 \times 10^{-4} \text{ s}^{-1}$.

Fig. 4. The pH-dependent optical absorption and resonance Raman spectra of ferric CcO
The optical absorption spectra are shown in panel (A) and the resonance Raman spectra are shown in panel (B). The excitation wavelength for the resonance Raman measurements was 413.1 nm from a Kr⁺ laser.

Fig. 5. The time-dependent optical spectra obtained following the pH jump from pH 7.4 to 12.0 in the ferric oxidation state

(A) The early time-dependent optical spectra obtained by stopped-flow measurements. (B) The slower spectral changes obtained by hand-mixing method with a deadtime of ~1 min. The spectra were acquired at 1, 12, 28, and 195 minutes after initiation of the reaction. The arrows indicate the trends for the changes in the optical intensity as the reaction progresses. The inset in (A) shows the kinetic traces obtained by a global fit of the time-dependent data associated with the $N_O \rightarrow N_O' \rightarrow I_O$ transition. The inset in (B) show the kinetic traces obtained by spectral deconvolution of the time-dependent data associated with the $I_O \rightarrow S_O$ transition. The smooth

grey line in the inset is a bi-exponential fit to the data with rate constants of 7×10^{-3} and $1 \times 10^{-3} \text{ s}^{-1}$.

Fig. 6. The standard spectra of N_O , N_O' and I_O
 The spectra were obtained from the global fit of the data shown in Fig. 5A.

Fig. 7. The time-dependent resonance Raman spectra obtained following the pH jump from pH 7.4 to 12.0 in ferric CcO (A) and the time evolution of the three resonance Raman modes as indicated (B)

The spectra associated with the $I_0 \rightarrow S_0$ transition were acquired at 1, 12, 28, and 195 minutes after initiation of the reaction by hand-mixing with a deadtime of ~ 1 min. The arrows indicate the trends for the changes in the intensity as the reaction progresses. The smooth black lines are single exponential fits to the data with rate constants as indicated.

Fig. 8. The time-dependent optical absorption spectra (A) and resonance Raman spectra (B) of ferric CcO obtained following the pH 10.2→12.0 jump

The spectra shown were acquired at 1, 2, 6, 16, 43, 74 and 96 minutes following the initiation of the reaction for (A) and at 1, 2, 6, 36 and 170 minutes for (B). The arrows indicate the trends for the changes in the spectra as the reactions progress. The inset in (A) shows the kinetic trace obtained by spectral deconvolution of the time-dependent data associated with the $I_0 \rightarrow S_0$ transition as described in the text. The smooth grey line is a bi-exponential fit to the data with rate constants of 3×10^{-3} and $0.3 \times 10^{-3} \text{ s}^{-1}$.

Fig. 9. The time-dependent optical absorption spectra of ferric CcO obtained following the pH 10.2→12.0 jump (A) and the standard spectra of L_O and I_O (B)
 The spectra in (A) were obtained by initiating the reaction in a stopped-flow system. The arrows indicate the trends for the changes in the optical intensity as the reactions progress. The inset shows the kinetic traces obtained by global fitting of the time-dependent data associated with the $L_O \rightarrow I_O$ transition.

Fig. 10. The time-dependent optical and resonance Raman spectra obtained following the pH jump from pH 7.4 to 10.2 in ferric CcO

The reaction was initiated by hand-mixing with a deadtime of ~ 1 min. The spectra listed in (A) were acquired at 1, 33, 58, and 301 minutes following the initiation of the reaction. The spectra listed in (B) were acquired at 1, 12, 28 and 195 minutes following the initiation of the reaction. The arrow indicates the trend for the change in the optical intensity as the reaction progresses. The inset in (A) shows the kinetic trace obtained by spectral deconvolution of the time-dependent data associated with the $N_O \rightarrow L_O$ transition as described in the text. The smooth grey line is a single exponential fit to the data with a rate constant of $2 \times 10^{-4} \text{ s}^{-1}$.

Fig. 11. The time-dependent optical absorption (A) and resonance Raman spectra (B) obtained following the dithionite reduction of the Schiff base derivative of the ferric CcO at pH 12.0
 The spectra shown were acquired at 0, 1, 2, 8 and 16 minutes following the initiation of the reaction for (A) and 1, 4, 8 and 90 minutes for (B). The arrows indicate the trends for the changes in the intensity as the reaction progresses. The inset in (A) shows the kinetic trace obtained by spectral deconvolution of the time-dependent data associated with the $S_O \rightarrow S_R$ transition as described in the text. The smooth grey line is a single exponential fit to the data with a rate constant of $1.8 \times 10^{-3} \text{ s}^{-1}$.

Table 1

Comparison of optical absorption and resonance Raman data from model heme *a* complexes and the various CcO derivatives from reference [28]. The absorbance maxima are expressed in nanometers and the Raman frequencies in wavenumbers.

Compound		Ferrous		Ferric	
		Abs	$\nu_{C=O}$	Abs	$\nu_{C=N}$
Model heme <i>a</i>	Aqueous (Bis-Im)	440, 597	1635	423, 595	1660
	CH ₂ Cl ₂ (Bis-Im)	438, 592	1644	424, 593	1667
Model heme <i>a</i> (Schiff base)	Aqueous (nBuNH ₂)	427, 573	-	416, 573	1643
	CH ₂ Cl ₂ (nBuNH ₂)	428, 575	-	418, 573	1640
CcO	heme <i>a</i> (pH 7.4)	443, 604*	1610	421, 601*	1647
	heme <i>a</i> ₃ (pH 7.4)	443, 604*	1664	421, 601*	1671
	Intermediate N _O '	-	-	420, 600*	1647/1671
	Species L _O	-	-	424, 599*	1647/1671
	Intermediate I _R	436, 594*	1634	-	-
	Intermediate I _O	-	-	411, 635*	1650-70
	Schiff base (pH 12.0)	428, 575*	-	407, 633*	1634

* The contributions from heme *a* and heme *a*₃ are indistinguishable.

Table 2

Rate constants for the pH jump transitions in CcO.

	Transition		Rate Constant (s^{-1})
Ferrous	$N_R \rightarrow I_R$	Native to bis-His	$k > 2 \times 10^{-2}$
	$I_R \rightarrow S_R$	Schiff Base formation	$k = 4 \times 10^{-4}$
Ferric	$N_O \rightarrow N_O'$	Conformational change	$k = 0.6$
	$N_O \rightarrow L_O$	His-Fe-(H ₂ O?) to His-Fe-OH	$k = 2 \times 10^{-4}$
	$N_O' \rightarrow I_O$	His-Fe-(H ₂ O?) to His-Fe	$k = 0.05$
	$L_O \rightarrow I_O$	His-Fe-OH to His-Fe	$k = 0.1$
	$I_O \rightarrow S_O$	Schiff Base formation *	$k = 5 \times 10^{-3}, 7 \times 10^{-4}$

* The rate constants for this transition is the average of the pH 7.4 to 12.0 jump and the 10.2 to 12.0 jump.