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Resolution of the electronic transitions of cytochrome *c* oxidase: Evidence for two conformational states of ferrous cytochrome *a*

(absorption spectroscopy/second derivative)

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ABSTRACT **Second-derivative absorption spectra are reported for a variety of oxidation and ligation states of bovine cytochrome *c* oxidase (ferrocytochrome-*c*:oxygen oxidoreductase, EC 1.9.3.1). The high resolving power of the second-derivative method allows us to assign the individual electronic transitions of cytochrome *a* and cytochrome a_3 in many of these states. In the fully reduced enzyme, one observes a single electronic transition at 444 nm, corresponding to the Soret transition for both ferrous cytochrome *a* and ferrous cytochrome a_3. When the cytochrome a_3 site is occupied by an exogenous ligand (CN or CO), one observes two absorption bands assignable to the ferrous cytochrome *a* chromophore, one at *ca.* 443 nm and the other at *ca.* 450 nm. The appearance of the 450-nm band is dependent only on ligand occupancy at the cytochrome a_3 site and not on the oxidation state of the cytochrome a_3 iron. These results can be interpreted either in terms of a heterogeneous mixture of two ferrous cytochrome *a* conformers in the cytochrome a_3-ligated enzyme or in terms of a reduction in the effective molecular symmetry of the ferrous cytochrome *a* site that results in a lifting of the degeneracy of the lowest unoccupied molecular orbital associated with the Soret π,π^* transition of cytochrome *a*. In either case, the present data indicate that ferrous cytochrome *a* can adopt two distinct conformations. One possible structural difference between these two states could be related to differences in the strength of hydrogen bonding between the ferrous cytochrome *a* formyl oxygen and a proton donor from an unidentified amino acid side chain of the enzyme. The implications of such modulation of hydrogen-bond strength are discussed in terms of possible mechanisms of proton translocation and electron transfer in the enzyme.**

Cytochrome *c* oxidase (ferrocytochrome-*c*:oxygen oxidoreductase, EC 1.9.3.1) is a redox-linked proton-translocating enzyme (1). While the identities of the structural elements involved in proton translocation have not been fully elucidated, it is clear that at least one of the four redox-active metal centers of the enzyme provides the thermodynamic driving force for proton pumping. To properly function as a redox-driven proton pump, the enzyme must be capable of adopting alternative protein conformations in each valence state of the involved metal center. These alternative conformations correspond to a proton-input state and a proton-output state of the enzyme in which a protonateable group(s) is provided alternative access to the two aqueous phases that are separated by the respiratory membrane (1–4). The ferric cytochrome a_3 cofactor is known to adopt two conformational states, referred to as resting and pulsed (2). Whether the resting state is accessible *in vivo* is, however, a matter of current debate. Recently, Ray *et al.* (5) have suggested that ferrous cytochrome a_3 can exist in either a five-coordinate high-spin or six-coordinate low-spin conformation within submitochondrial particles. Thus, conformational flexibility of the ligand-binding heme of cytochrome *c* oxidase seems well established. However, there is a significant body of data that suggests a role for one of the low-potential metal centers of the enzyme in proton translocation (1–4). Two structural models for proton pumping by cytochrome *c* oxidase have emerged, one involving redox-linked changes in hydrogen bonding at cytochrome *a* (4) and the other involving redox-linked ligand rearrangements at Cu_A (3). At present, there is no experimental basis for ruling out either of these hypotheses.

One of the most characteristic features of heme prosthetic groups is a strongly allowed π,π^* transition of the conjugated porphyrin ring system which occurs in the 350- to 450-nm region of the electronic spectrum (often referred to as the Soret transition) (6). The exact wavelength maximum for this transition varies depending on the peripheral substituents on the porphyrin ring and the valence state of the central metal ion; it is also modulated by the details of the protein structure in which the heme is embedded (i.e., electrostatic interactions, hydrogen bonding, and dielectric constant of the surrounding medium). Despite the presence of two heme chromophores, however, the absorption spectrum of resting cytochrome *c* oxidase (i.e., as isolated, with all four redox-active metals in their oxidized valence states) is anomalous in that only a single Soret band is observed at *ca.* 421 nm, even at cryogenic temperatures (7). This lack of spectral resolution of the two hemes persists in many of the redox and liganded states of the enzyme. Since much of our knowledge of the structure and reactivity of the heme groups of cytochrome *c* oxidase is based on steady-state and kinetic-absorption measurements, the resolution of the contributions of the individual heme groups to the absorption spectrum of the enzyme becomes a critical issue (2).

In this study we experimentally resolved the electronic transitions of the two hemes of cytochrome *c* oxidase by determining the second derivative of the absorption spectrum of the enzyme in various redox and ligation states. The data presented here suggest that ferrous cytochrome *a* can adopt two alternative conformations, depending on whether the other heme (cytochrome a_3) is coordinated by an exogenous ligand. These results support the view that cytochrome *a* and cytochrome a_3 are in allosteric communication with one another. The two ferrous cytochrome *a* conformers could potentially play a role in a mechanism of redox-coupled proton pumping by cytochrome *c* oxidase in which the cytochrome *a* chromophore serves as the coupling site between electron transfer and proton translocation.

MATERIALS AND METHODS

Bovine cytochrome *c* oxidase was isolated from cardiac muscle as described (8). *Paracoccus denitrificans* (American

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Type Culture Collection, ATCC 13543) were grown aerobically in 60-liter batches and purified as described by Ludwig and Schatz (9). For optical spectroscopy, the enzymes were thawed on ice and diluted with 50 mM Hepes buffer (pH 7.4) containing 0.1% Tween 20 (Pierce) to a concentration yielding an optical density of *ca.* 1.4 at the Soret maximum, except for the *P. denitrificans* enzyme, which was diluted to an optical density of 0.7 because of limited supplies. The final concentrations of the enzyme samples were determined spectrophotometrically by using the following extinction coefficients: resting bovine enzyme, $\varepsilon_{421\ nm} = 141\ mM^{-1}\cdot cm^{-1}$ (10); cyanide (CN)-inhibited bovine enzyme, $\varepsilon_{429\ nm} = 156\ mM^{-1}\cdot cm^{-1}$ (10); and resting *P. denitrificans* enzyme, $\Delta\varepsilon_{424-480\ nm} = 60.1\ mM^{-1}\cdot cm^{-1}$ (9).

Conformational states of the enzyme that are sensitive to air oxidation were prepared under a nitrogen atmosphere in an anaerobic cuvette. The various stable forms of the enzyme were prepared by procedures described in the following references: resting, fully reduced, CN-inhibited, reduced CN-inhibited, and CN mixed-valence states (11); 428-nm (pulsed-peroxide) and 420-nm (pulsed) states (12); CO-bound reduced and CO-bound mixed-valence states (13); and the oxyferryl state (14).

Optical spectra were recorded at 25°C with a Cary 14 UV–Visible–near IR spectrophotometer. The instrument was interfaced to an IBM-compatible computer that 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 bandpass of 0.5 nm. Each reported spectrum is the average of 10 such scans. The second derivative of each absorption spectrum ($\partial^2 A/\partial\lambda^2$) was obtained by the least-squares method of Savitzky and Golay (15).

RESULTS

Fig. 1 illustrates the absorption spectrum of resting-state bovine cytochrome *c* oxidase and the second-derivative spectrum derived from the parent spectrum. The absorption spectrum seen here is typical for the enzyme from a variety of eukaryotic and prokaryotic species. It displays a single, featureless absorption band centered at 421 nm. Undoubtedly this absorption-band envelope contains the unresolved contributions of both cytochrome *a* and cytochrome a_3. Indeed, the second-derivative spectrum for this enzyme shows clear evidence of two absorbing species. The higher energy component displays a wavelength maximum of 416 nm, while the lower energy transition occurs at 429 nm. Similar results were obtained for the resting state of cytochrome *c* oxidase from *P. denitrificans*, except that the higher energy transition occurred at 414 instead of 416 nm in the bacterial enzyme. Vanneste (16) has computed the absolute absorption spectra of cytochrome *a* and cytochrome a_3 in resting cytochrome *c* oxidase on the basis of the "ligand method." This analysis indicates that cytochrome a_3 has an absorption maximum at 414 nm, while cytochrome *a* has an absorption maximum at 426 nm. It is interesting to note that the absorption maxima computed by Vanneste are in close agreement with the absorption maxima observed in the second-derivative spectrum of the resting enzyme.

Fig. 2 illustrates the second-derivative absorption spectra of fully reduced (i.e., a^{2+},a_3^{2+}), CO mixed-valence (i.e., a^{3+},a_3^{2+}—CO), and reduced CO-bound (i.e., a^{2+},a_3^{2+}—CO) forms of the enzyme. In the fully reduced form, one observes a single band at *ca.* 444 nm, which represents the Soret electronic transition of both ferrous cytochrome *a* and cytochrome a_3. The CO mixed-valence form likewise shows a single band at 429 nm, representing the Soret transitions for

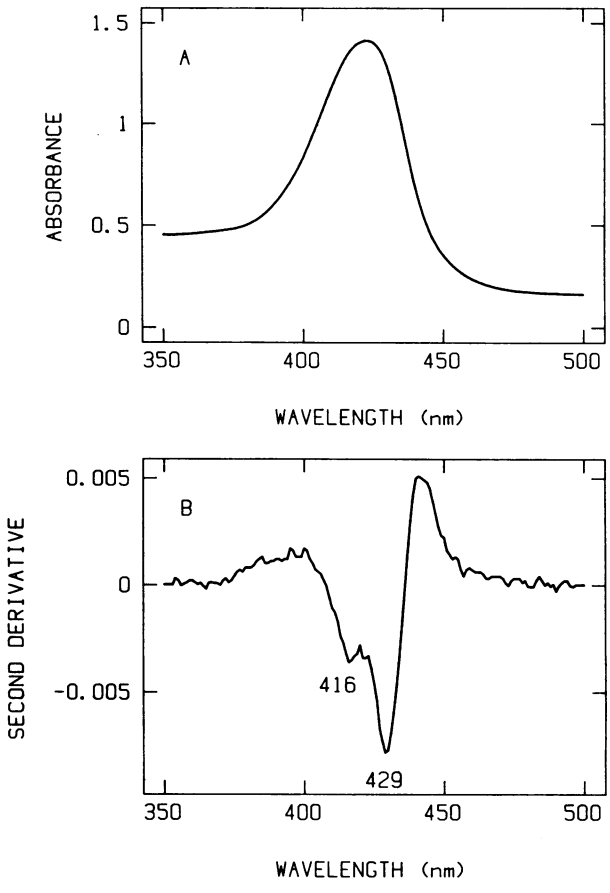

FIG. 1. Absorption (*A*) and second-derivative (*B*) spectra of resting-state bovine cytochrome *c* oxidase, indicating the locations of the resolved electronic transitions of cytochrome *a* and cytochrome a_3.

ferrous, six-coordinate, low-spin cytochrome a_3 and ferric, six-coordinate, low-spin cytochrome *a*. Reduction of cytochrome *a* in the CO-bound enzyme is expected to shift the Soret transition of this heme from 429 nm to *ca.* 444 nm. Thus, in the reduced CO-bound enzyme, one would expect to see two bands in the second-derivative spectrum—at 429 nm (ferrous, CO-bound cytochrome a_3) and 444 nm (ferrous cytochrome *a*). As seen in Fig. 2*C*, however, in this state of the enzyme one observes three bands in the second-derivative spectrum—the two expected bands and a third band at 451 nm. Comparison of the three spectra presented in Fig. 2 suggests that the 451-nm feature is associated with reduction of cytochrome *a* when cytochrome a_3 is ligated by an exogenous ligand. This inference is confirmed by study of the CN-inhibited enzyme, as illustrated in Fig. 3. Here we show the second-derivative absorption spectra of the oxidized, CN-inhibited (i.e., a^{3+},a_3^{3+}—CN), mixed-valence CN-inhibited (i.e., a^{2+},a_3^{3+}—CN), and fully reduced, CN-inhibited (i.e., a^{2+},a_3^{2+}—CN) enzyme. In the oxidized, CN-inhibited enzyme, both hemes are six coordinate and low spin. The second-derivative spectrum of this form of the enzyme therefore displays a single Soret band at 430 nm. In the mixed-valence form, the low-spin, ferric cytochrome a_3 transition is still observed at 428 nm, but the ferrous cytochrome *a* chromophore gives rise to two bands at 442 and 450 nm. Reduction of cytochrome a_3 prior to CN binding results in a second-derivative spectrum that is dominated by two bands at 443 nm (ferrous cytochrome a_3 and cytochrome *a*) and 450 nm (cytochrome *a*).

We have also obtained second derivative absorption spectra for a number of stable forms of cytochrome *c* oxidase that

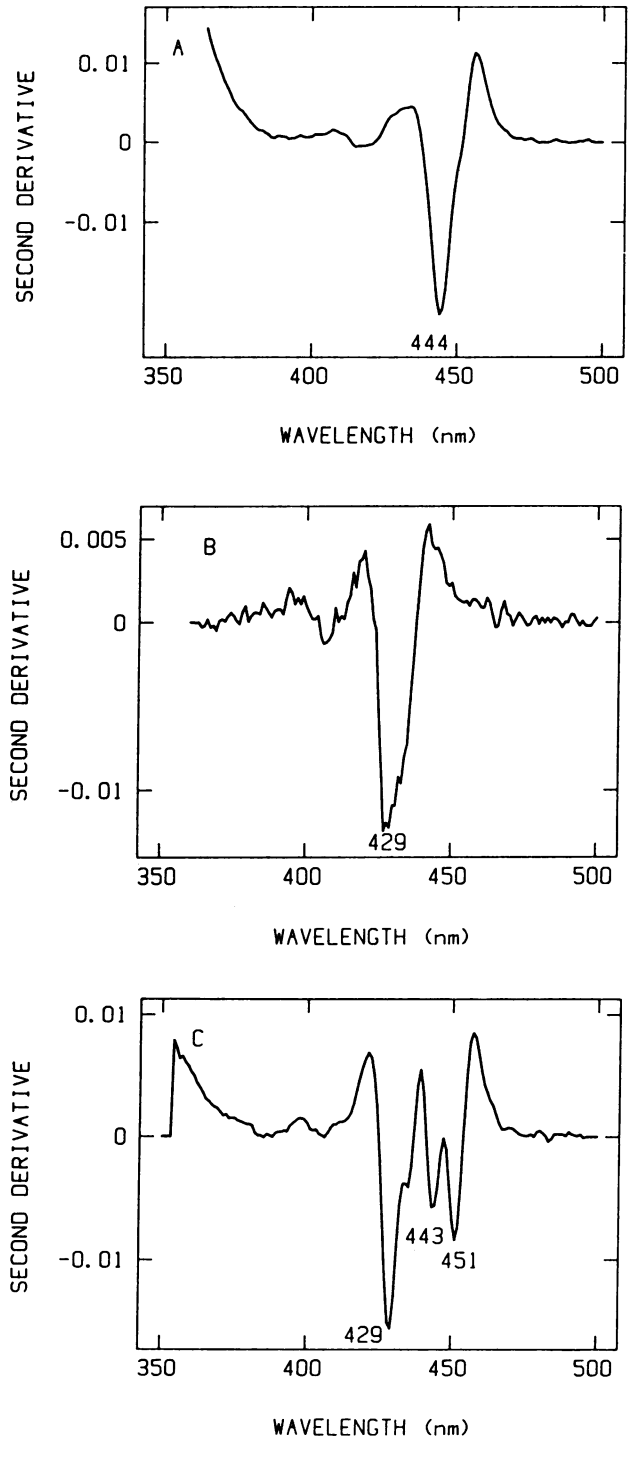

FIG. 2. Second-derivative absorption spectra of bovine cytochrome *c* oxidase in the fully reduced (*A*), CO-bound mixed-valence (*B*), and CO-bound reduced (*C*) forms.

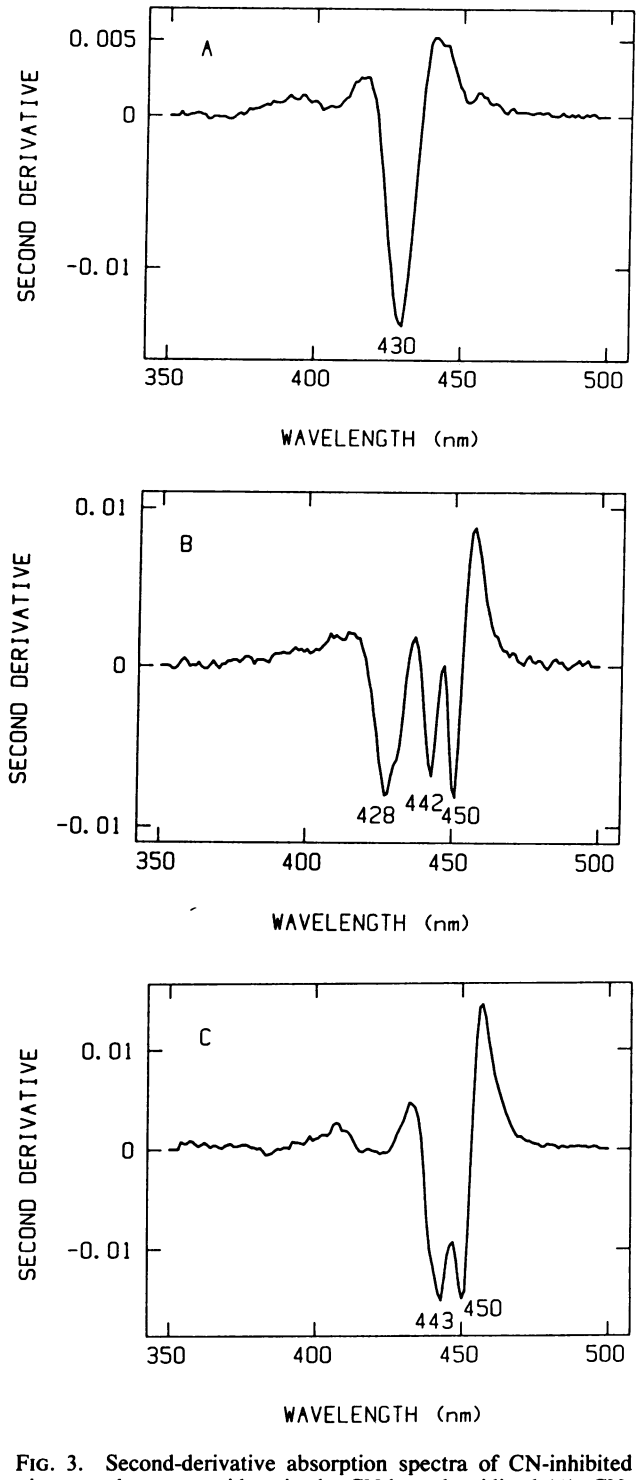

FIG. 3. Second-derivative absorption spectra of CN-inhibited bovine cytochrome *c* oxidase in the CN-bound oxidized (*A*), CN-bound mixed-valence (*B*), and CN-bound fully reduced (*C*) forms.

are thought to represent intermediates in the electron-transfer cycle of this enzyme, such as the oxyferryl and pulsed-peroxide states. The transition wavelengths and derivative extinction coefficients ($\partial^2\varepsilon/\partial\lambda^2$) for these various enzyme forms are summarized in Table 1.

DISCUSSION

The most surprising result from the present work is that there appear to be two conformational states accessible to ferrous cytochrome *a*, and the transition between these states is modulated by ligand-binding events at cytochrome a_3. The second-derivative spectra show clear evidence for two Soret transitions for ferrous cytochrome *a* in states of the enzyme in which cytochrome a_3 is ligated. The fact that the same two cytochrome *a* transitions are seen in the fully reduced CO-bound and CN-bound mixed-valence enzyme suggests that the conformational transition of cytochrome *a* is dependent only on ligand occupancy at cytochrome a_3 and not on the oxidation state of the ligand-binding heme. When the present experiments were repeated with the enzyme from *P. denitrificans*, similar results were obtained (data not shown). Interestingly, Wilson and Gilmour (17) have observed a similar splitting of the ferrous cytochrome *a* Soret band in the

Table 1. Soret transition maxima and derivative extinction coefficients for various states of bovine cytochrome *c* oxidase from second-derivative absorption spectroscopy

Enzyme state	Heme electron distribution	λ_{max}, nm ($\partial^2\varepsilon/\partial\lambda^2$, $M^{-1}\cdot cm^{-1}$)
Resting	a^{3+},a_3^{3+}	416 (330), 429 (726)
Fully reduced	a^{2+},a_3^{2+}	444 (2417)
CN-bound	a^{3+},a_3^{3+}—CN	430 (1538)
Mixed valence	a^{2+},a_3^{3+}—CN	428 (787), 442 (679), 450 (806)
Reduced	a^{2+},a_3^{2+}—CN	443 (1189), 450 (1173)
CO-bound		
Reduced	a^{2+},a_3^{2+}—CO	429 (1950), 443 (713), 451 (1050)
Mixed valence	a^{3+},a_3^{2+}—CO	429 (1550)
Pulsed (420-nm form)	a^{3+},a_3^{3+}	416 (336), 430 (595)
Pulsed-peroxide (428-nm form)	a^{3+},a_3^{3+}—OO—	428 (875)
Oxyferryl	a^{3+},a_3^{4+}=O	428 (939)

cryogenic (77 K) reduced-minus-oxidized difference spectrum of rat liver mitochondria in the presence of CN or sulfide. Thus, the two conformations of ferrous cytochrome *a* are preserved across tissue and species (as far as has been tested), indicating that the availability of two conformational states for this chromophore may be important for proper cytochrome *c* oxidase functioning. Since the enzyme from *P. denitrificans* is known to be monomeric under the conditions used here (18), the observation of two ferrous cytochrome *a* Soret bands in ligated forms of this enzyme indicates that the present results cannot be accounted for by inhomogeneity of the two cytochrome *a* groups in the dimeric mammalian enzymes.

That the oxygen-binding site and cytochrome *a* are in allosteric communication has been assumed for some time on the basis of several experimental results, including changes in the kinetics of CN binding upon reduction of cytochrome *a*, changes in the pH dependence of the redox potential cytochrome *a* when CN is bound to cytochrome a_3, etc. (19). The present results provide direct evidence for modulation of the cytochrome *a* conformation by cytochrome a_3 ligand binding.

Splitting of the Soret transitions for ferrous cytochrome *a* in ligand-bound forms of the enzyme (384 ± 29 cm^{-1}) could arise from either of two effects. First, the two bands could represent the individual Soret transitions for two discrete conformational states of cytochrome *a* that are in equilibrium with one another in ligand-bound forms of the enzyme. One of these conformers absorbs at *ca.* 443 nm and is the dominant species present in the unligated forms of the enzyme. The second conformer absorbs at *ca.* 450 nm; ligand binding to cytochrome a_3 results in a partial conversion of ferrous cytochrome *a* to this state. The Soret maximum for ferrous cytochrome *a* in the unligated enzyme (443 nm) is anomalously red-shifted relative to heme A model compounds. This red shift has been suggested to arise from hydrogen bonding of the cytochrome *a* formyl oxygen to a nearby proton-donating amino acid side chain (20). Babcock and Callahan (20) have quantified the enthalpy of hydrogen bonding (ΔH_{HB}) for the cytochrome *a* formyl group from the red shift of the absorption bands of the enzyme and model compounds relative to the reference spectrum. The further red shifting of the 450-nm ferrous cytochrome *a* Soret band could be a consequence of strengthening of the formyl hydrogen bonding in this state of the enzyme. Other factors, such as changes in electrostatic interactions about the heme, may also be contributing to the observed red shift (20).

The existence of two ferrous cytochrome *a* states in the ligated enzyme offers a potential explanation for the anomalous spectroelectrochemical titration behavior of the CN-inhibited enzyme. Several groups have reported that the titration of cytochrome *a* follows the expected $n = 1$ Nernstian behavior for the unligated enzyme, but in the CN-inhibited enzyme this chromophore titrates as an $n = 0.5$ redox center (2). Wikström *et al.* (2) have suggested that this behavior could be accounted for by an inhomogeneity of the redox behavior of cytochrome *a*. This inhomogeneity could arise from two alternative conformations of cytochrome *a* and/or from anticooperativity between the redox potentials of cytochrome *a* and Cu_A. Ellis *et al.* (21) have recently interpreted the spectroelectrochemical behavior of cytochrome *a* and Cu_A in terms of such anticooperativity. However, this behavior also could arise from the combined effects of anticooperativity between the two low potential metal centers and conformational heterogeneity of cytochrome *a*.

An alternative explanation for the observation of two ferrous cytochrome *a* Soret bands is that ligation at cytochrome a_3 results in a conformational transition that lowers the effective symmetry of the cytochrome *a* chromophore. For heme groups with symmetric (or quasi-symmetric) placement of peripheral groups on the tetrapyrrole ring system, the electronic spectrum reflects D_{4h} effective symmetry (22, 23). For heme A, the asymmetric placement of peripheral groups on the porphyrin should lead to a lowering of symmetry to D_{2h}. In this point group, the degeneracy of the E_g lowest unoccupied molecular orbital should be lifted, resulting in two Soret transitions, one $A_u \rightarrow B_{2g}$ and one $A_u \rightarrow B_{3g}$ (23). While the effects of this symmetry lowering have been observed for the vibronically allowed electronic transitions, no evidence for splitting of the Soret transition of heme A has been observed (23). If, however, the symmetry were further lowered by specific interactions with the protein, one might expect this splitting to be observed. Again, strengthening of the formyl hydrogen bond would be one effective means of producing this symmetry lowering. Stronger hydrogen bonding at the formyl group would pull electron density away from the porphyrin ring selectively along one of the two symmetry axes within the plane of the ring system, as illustrated in Fig. 4. While the energy gap for the two π^* states of heme A under D_{2h} symmetry has not been experimentally determined, we note that the Soret splitting observed here (*ca.* 384 cm^{-1}) is of the same magnitude as that observed for other low-spin ferrous heme proteins in single-crystal polarized absorption spectra (22).

Regardless of which of the above explanations is correct, the second-derivative absorption data clearly indicate the presence of two ferrous cytochrome *a* conformers. We note that the existence of two states of cytochrome *a* has been inferred previously on the basis of a number of kinetic experiments and was predicted in the proton-pumping model

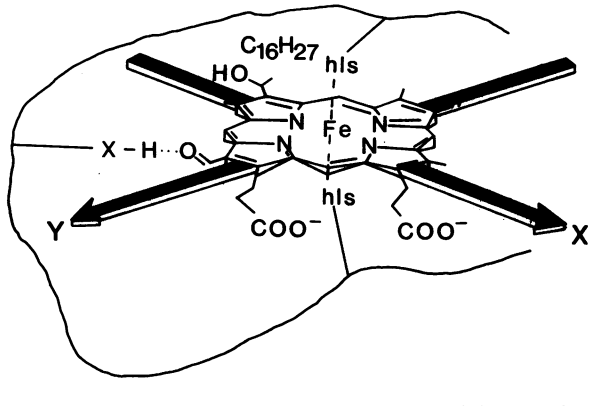

FIG. 4. Structural model adapted from ref. 4 of the cytochrome *a* chromophore of cytochrome *c* oxidase showing the psuedo-synmmetry axes within the plane of the porphyrin ring system.

of Wikström *et al.* (2). However, the present results provide direct experimental evidence for two states of this chromophore. It is tempting to speculate on what the enzymatic significance of these two conformers might be. It is widely accepted that one of the low-potential metal centers of cytochrome *c* oxidase, either cytochrome *a* or Cu_A, is involved in redox-coupled proton translocation. Babcock and Callahan (4) have suggested that cytochrome *a* is the metal cofactor involved in proton pumping and that the coupling is facilitated by redox-induced changes in cytochrome *a* formyl hydrogen bonding to an amino acid side chain. This model requires four conformations of cytochrome *a* to complete a proton translocation cycle, two ferric states and two ferrous states. It is thus possible that the results presented here reflect the two ferrous cytochrome *a* states required by the Babcock model. Alternatively, the two ferrous cytochrome *a* conformers could provide a means of controlling internal electron transfer between cytochrome *a* and the oxygen binding site. Malmström (19) has recently presented data that suggest that electron transfer from the low-potential metal centers to the oxygen-binding site is rate-limited by a conformational transition that occurs only after the two low-potential centers are saturated with electrons. Perhaps the two conformers that we have detected here represent an electron transfer-competent state and an alternative state that does not transfer electrons to the oxygen-binding site.

Continued investigation of the second-derivative spectra of ferrous cytochrome *a* forms of the enzyme is clearly warranted. In particular, kinetic and steady-state studies of vesicle-reconstituted enzyme may help to resolve some of the issues raised in the present study.

Note. While this paper was under review we obtained second-derivative spectra of the bovine enzyme during steady-state turnover. The results of these studies suggest that the 450 nm-absorbing form of cytochrome *a* is the dominant species present during enzyme turnover (R.A.C., unpublished data).

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