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Long-Distance Cofactor Interactions in Terminal Oxidases Studied by Second-Derivative Absorption Spectroscopy[1]

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The electronic transitions of the two heme groups of cytochrome *c* oxidase have been resolved by application of second-derivative and cryogenic absorption spectroscopy. Both methods reveal a splitting of the ferrocytochrome *a* Soret transition into two features at 443 and 450 nm. The relative intensity of the 450 nm feature appears to depend on the ligation state of cytochrome a_3, the solution pH, and complex formation with cytochrome *c*. The structural origin and mechanistic significance of this second Soret transition of cytochrome *a* are discussed in terms of the electron transfer and proton translocation activities of the enzyme.

KEY WORDS: Cytochrome oxidase; spectroscopy; oxidative phosphorylation.

INTRODUCTION

The terminal oxidases of aerobic respiration constitute a family of structurally related, multisubunit enzymes that each catalyze the reduction of molecular oxygen to water, and the concomitant translocation of protons across the respiratory membranes (Gennis, 1991). As illustrated in Fig.1, these varied enzymes share in common certain structural elements that facilitate their common enzyme activities. All of these enzymes contain a bis-histidine-coordinated heme group that serves as an electron conduit to the site of molecular oxygen binding and reduction. The oxygen binding site itself consists of two metal cofactors, a 5-coordinate heme and a copper ion (Cu_B), that are in close spatial proximity ($\leqslant 5$ Å) and together provide the loci for binding, reduction, and homolytic bond cleavage of dioxygen (Wikström *et al.*, 1981). All three of these metal cofactors are contained within the largest polypeptide subunit of the enzymes, subunit I. A subgroup of enzymes in this family contains a second copper site (Cu_A), within subunit II, that serves as an entry point into the enzyme for electrons from cytochrome *c*; these enzymes are known as cytochrome *c* oxidases, to denote the fact that the physiological electron source for them is ferrocytochrome *c*. Those

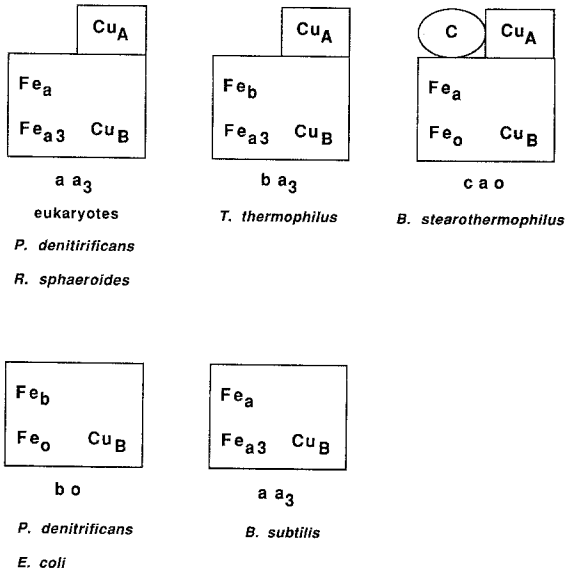

Fig. 1. Schematic representation of the structural variety within the superfamily of terminal oxidases.

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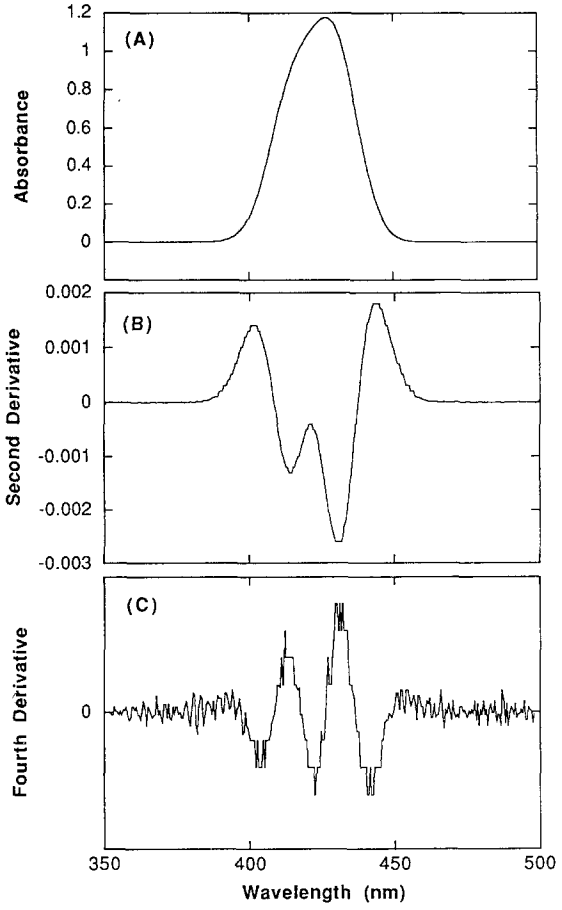

Fig. 2. Resolution enhancement by derivatization of absorption spectra. Panel A shows a simulated absorption spectrum that results from the combination of two Gaussian bands with peak centers at 415 and 430 nm. Both bands have equal bandwidths at half maximum (8 nm), but the extinction coefficient of the lower-wavelength band is only 0.75 times that of the higher-wavelength band. Panel B shows the second derivative of the composite spectrum from Panel A. Panel C shows the corresponding fourth-derivative spectrum.

members of the family that lack Cu_A derive their electron equivalents directly from ubiquinol *in vivo*; they are thus known as ubiquinol oxidases.

For all of the enzymes in this family, a requisite step in their catalytic mechanisms is intramolecular electron transfer from the low-spin, six-coordinate heme to the binuclear oxygen binding center. The rate of this intramolecular electron transfer event varies greatly with experimental conditions. For example, in vesicle-reconstituted systems, the rate of intramolecular electron transfer is strongly dependent on the electrochemical potential gradient across the phospholipid bilayer, a phenomenon known as respiratory control (Nicholls, 1982). It has been suggested that the control of intramolecular electron transfer (referred to as electron gating), by structural rearrangements of the protein, may play an important role in coordinating the electron transfer and proton translocation activities of these enzymes (Wikström *et al.*, 1981).

Communication between the two heme centers of these enzymes is manifested not only in controlled electron transfer, but in other characteristics of the active enzymes as well. Despite a separation of some 19 Å, the reduction potentials of the two hemes are interdependent in an anticooperative fashion (Artzatbanov *et al.*, 1978). Likewise, the rate of inhibitory ligand binding at the binuclear center is greatly accelerated by electron occupancy at the low-spin heme (Andreev *et al.*, 1983). What structural elements facilitate this type of long-distance communication between the heme groups of these enzymes, and what mechanistic roles might such communication fulfill? To answer these questions, one would wish to examine the physicochemical properties of the two heme groups independently, as reporters of structural alterations of the hemes themselves and of the surrounding protein, under conditions of physiological relevance. Since the energy and intensities of π–π^* electronic transitions of the hemes are sensitive to structure and environment, optical spectroscopy is an obvious choice for exploring transitions in the vicinity of these cofactors. Unfortunately, however, the two heme groups most oxidases are chemically identical and, despite their differing environments, do not give rise to resolvable absorption bands under most experimental conditions.

To help ameliorate this spectral resolution problem, and begin to systemically explore the structural transitions that attend enzyme activity, our group has recently applied second-derivative absorption spectroscopy to these systems. Focusing on the subgroup of enzymes that contains the heme A prosthetic group, we have been able to resolve the individual transitions of the cytochrome *a* (low-spin heme) and cytochrome a_3 (binuclear-center heme) cofactors of the cytochrome *c* oxidases using this method (Sherman *et al.*, 1991). Some surprising information on heme–heme communication has been gleaned by this approach. The data that will be reviewed here suggest that the cytochrome *a* cofactor gives rise to two π–π^* transitions in the Soret region of the electronic spectrum. The relative intensities of these two transitions appear to be affected by ligand binding at the binuclear center, and by cytochrome *c* binding on subunit II. One

of these π–π^* transitions has not previously been resolved in room-temperature spectra of the enzyme, and appears to be unique to cytochrome *a* within the protein environment of the native enzyme.

SECOND-DERIVATIVE SPECTROSCOPY

The approach to enhanced spectral resolution that we have taken is to obtain the optical spectrum of the enzyme in terms of absorbance as a function of wavelength, and to then calculate the second derivative of absorbance with respect to wavelength. The basis for this approach is illustrated in Fig. 2. When one has closely lying absorption bands that are unresolved in the parent spectrum, one can often resolve these bands by computing the *n*th derivative of absorbance with respect to wavelength. As the derivative order *n* increases, the band widths associated with individual spectral components decreases, thus leading to enhanced resolution. If the derivative order is even, then transition maxima in the parent spectra will appear as extremes (either minima or maxima) in the derivative spectra. In Fig. 2A we have composed an absorption spectrum from two closely lying Gaussian bands, one maximizing at 415 nm and the other at 430 nm. Both bands have bandwidths at half maximum of 8 nm, and the lower-wavelength component has an extinction coefficient that is 0.75 times that of the higher-wavelength component. With these parameters, the two bands are not resolved in the parent spectrum. In panel B we show the second derivative of this composite absorption spectrum, and observe that here the two bands are clearly resolved; the bands appear as minima in the second-derivative spectrum, but faithfully report the wavelengths of the unresolved peaks from the parent spectrum. As seen in panel C, the bands are even better resolved in the fourth-derivative spectrum, and here the bands once again appear as maxima. Panel C also illustrates the fact that as the derivative order increases, there is a steady reduction in the signal-to-noise ratio for the data. In many cases the resolution afforded by the second derivative is sufficient, and the reduction in data quality is minimal.

The absorption spectrum of resting (i.e., as isolated with all metal cofactors oxidized) cytochrome *c* oxidase is typical of a heme-containing protein, displaying a strong π–π^* transition between 400 and 500 nm, known as the Soret transition (Fig.3A). Although the absorption spectrum of the enzyme appears as a single band, the individual transitions of the

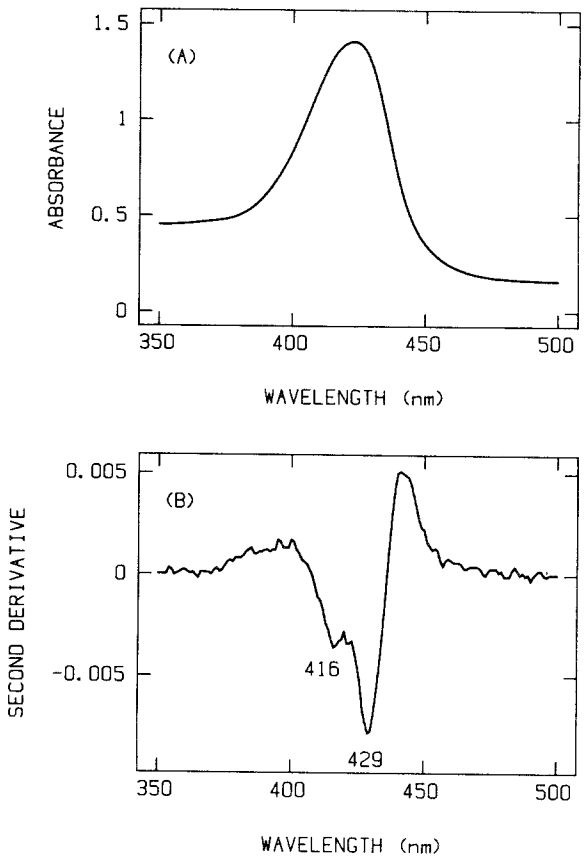

Fig. 3. Absorption (A) and second derivative (B) spectra of resting bovine cytochrome *c* oxidase in the Soret band region. Data taken from Sherman *et al.* (1991).

two hemes must be contained within this band envelope. On the basis of "computational methods" Vannest (1966) has predicted that the Soret maxima for cytochrome *a* and cytochrome a_3 should occur at 426 and 414 nm, respectively. When the absorption spectrum in Fig. 3A is analyzed by second-derivative methods, one observes clear evidence for two transitions at 416 and 429 nm (Fig.3B), in remarkably good agreement with Vanneste's predictions. That the 416 nm band is assignable to cytochrome a_3 is confirmed by the fact that this band is lost when the enzyme is incubated with cyanide, conditions under which the 5-coordinate, high-spin cytochrome a_3 is converted to a 6-coordinate, low-spin state (Sherman *et al.*, 1991).

EVIDENCE FOR TWO SORET TRANSITIONS OF FERROCYTOCHROME *a*

Having established that we could resolve the

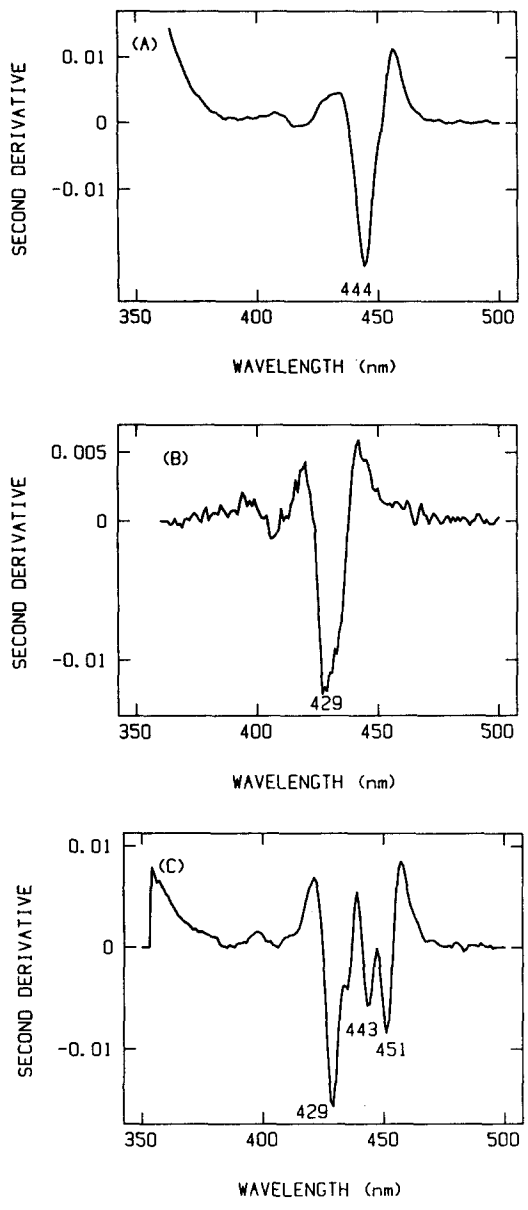

Fig. 4. Second-derivative absorption spectra of bovine cytochrome *c* oxidase in the fully reduced unliganded (A), CO-bound mixed-valence (B), and CO-bound reduced (C) forms. Data taken from Sherman *et al.* (1991).

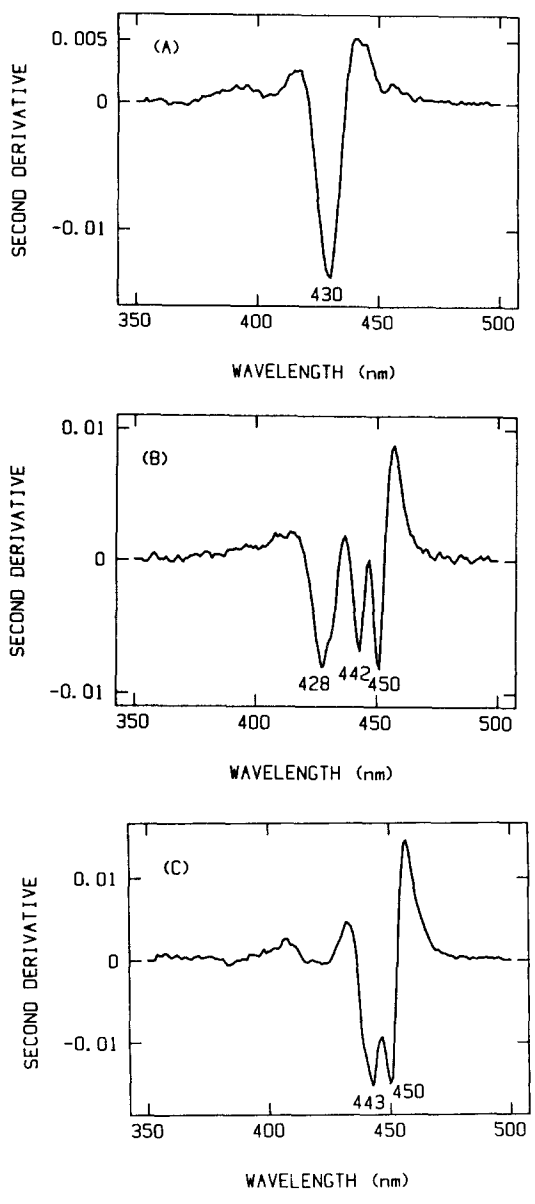

Fig. 5. Second-derivative spectra of CN-inhibited bovine cytochrome *c* oxidase in the oxidized (A), CN-bound mixed-valence (B), and CN-bound reduced (C) forms.

Soret transitions of the two heme groups of cytochrome *c* oxidase by second-derivative spectroscopy, we next explored the second-derivative spectra of stable variants of the mammalian enzyme. Figures 4 and 5 illustrate the second-derivative spectra of various stable forms of the bovine enzyme. The most important new information obtained from these studies is that there is an electronic transition at ca. 450 nm which is best observed when cytochrome *a* is reduced and cytochrome a_3 is ligated by an exogenous ligand (i.e., CO or CN). We have suggested that this 450 nm band is a second Soret transition of ferrous cytochrome *a* that represents an alternative conformation of the protein pocket surrounding this cofactor (Lynch and Copeland, 1992). Careful inspection of the second derivative spectrum of fully reduced, unliganded cytochrome *c* oxidase (Fig. 4A) reveals

that there is a weak 450 nm component in this spectrum as well. We have confirmed that the 450 nm band contributes to the absorption spectrum of the fully reduced unliganded enzyme by studying the *aco* terminal oxidase from *Bacillus* YN-2000. In collaboration with Professor Tateo Yamanaka (Tokyo Institute of Technology) and his group, we have obtained second-derivative absorption spectra of the fully reduced unliganded form of this enzyme, in which the cytochrome a_3 heme is replaced with a heme *o* group. The data reveal clear evidence for a 450 nm band in this enzyme form (Horvath *et al.*, unpublished data). Thus, even in the unliganded state of the enzyme, cytochrome *a* displays two Soret transitions. However, the 450 nm transition is intensified, and most clearly observed upon ligand occupancy at the binuclear center. The apparent intensification of this band is dependent solely on ligand binding at the binuclear site, and not on the valence state of cytochrome a_3. Thus, one observes this band in the CN-inhibited mixed valence (i.e., a^{2+}, a_3^{3+}-CN) and CN-inhibited reduced (i.e., a^{2+}, a_3^{2+}-CN) enzymes, as well as in the reduced CO-inhibited enzyme (i.e., a^{2+}, a_3^{2+}-CO). We have interpreted these data as indicating a shift in equilibrium in favor of the 450 nm-absorbing conformer of cytochrome *a*, when ligands bind to the binuclear site. In other words, ligand binding at a site 19 Å away effects a conformational adjustment of the protein surrounding cytochrome *a*; such a transmission of a conformational transition between the two heme sites, via the intervening protein, would provide a mechanistic explanation for the indirect evidence of inter-heme communication discussed above.

An alternative interpretation of these data is possible, however, which does not require the presence of two conformational states of ferrous cytochrome *a*. It is known that the π^* state associated with the Soret transition of hemes is degenerate. Upon symmetry lowering, this degeneracy should be lifted so that the single Soret transition would split into two separate π–π^* transitions. This band splitting is expected for the heme A group simply because of the asymmetric placement of peripheral groups on the porphyrin ring system, but has not previously been observed. If the protein pocket surrounding cytochrome *a* somehow lowers the symmetry of this group further, such splitting might be observed. In this interpretation the 443 and 450 nm bands would represent the *x* and *y* polarized components of the usually degenerate Soret transition (Sherman *et al.*, 1991). The apparent intensification of the 450 nm band in liganded forms of the enzyme might simply be due to the removal of cytochrome a_3 absorption contributions to this spectral region, thus unmasking the true intensity of the 450 nm feature (we wish to acknowledge that this latter interpretation was brought to our attention by Dr. Denis L. Rousseau). On the basis of the available experimental data, we cannot at present rule out either of these interpretations.

THE 450 nm BAND IS OBSERVED DURING ENZYME CATALYSIS

The 450 nm electronic transition not only appears when the enzyme is inhibited by exogenous ligands, but also appears when the natural substrate O_2 binds to the enzyme (Copeland, 1991). We have addressed this issue by analyzing the second-derivative spectrum of the bovine enzyme during steady-state turnover. Using cytochrome *c* and molecular oxygen as substrates, we initiated enzyme turnover by the addition of sodium ascorbate to provide electron equivalents to cytochrome *c*. When resting cytochrome *c* oxidase and ferricytochrome *c* are mixed with oxygen-saturated buffer, the ferricytochrome *a* transition occurs at 430 nm in the second-derivative spectrum. Addition of ascorbate leads to reduction of cytochrome *c* which triggers steady-state turnover of the oxidase until the solution is exhausted of oxygen. During steady-state turnover, one observes the ferrocytochrome *a* transition maximum at 450 nm, but there may be an unresolved shoulder at 443 nm as well (these spectra were obtained with a diode array detector which has limited spectral resolving power). Once the dissolved oxygen has been depleted by the enzyme, the system becomes anaerobic and both cytochrome *c* and the enzyme assume their static reduced conformations; in this state the electronic transitions for ferrocytochrome *a* and ferrocytochrome a_3 are observed at 444 nm. Thus, during steady-state turnover the enzyme exhibits a transition at 450 nm when oxygen is bound to the binuclear center and cytochrome *a* is reduced.

THE 450 nm BAND IS OBSERVED IN LOW-TEMPERATURE ABSORPTION SPECTRA

To establish that the features observed in the

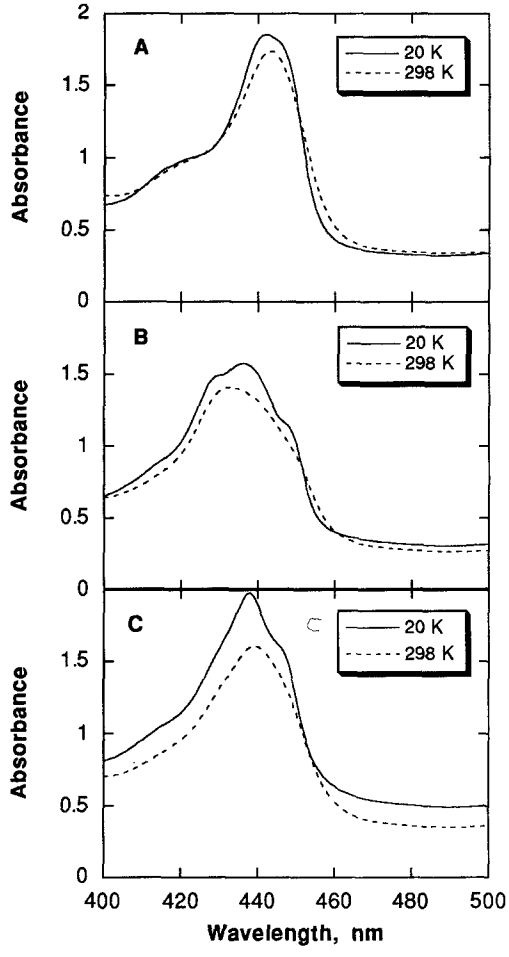

Fig. 6. Absorption spectra of bovine cytochrome *c* oxidase at 298 K (dashed lines) and 20 K (solid lines) in the reduced unliganded (A), CO-bound reduced (B), and CN-bound reduced (C) forms.

second-derivative spectra represent genuine transitions of the hemes, we have looked to more traditional means of enhancing the resolution of the electronic spectra. A common means of enhancing resolution is to work at lower temperatures where homogeneous line broadening is minimized. We have therefore obtained spectra of the reduced enzyme forms in glycerol glasses at temperatures between 298 and 20 K. A detailed description of the effects of lowered temperature on the electronic transitions of the enzymes from bovine heart and *Paracoccus denitrificans* will be presented shortly (Horvath *et al.*, unpublished data). Here we illustrate (Fig. 6) the absorption spectra of reduced unliganded (A), CO- (B), and CN-bound (C) bovine oxidase at the two temperature extremes, 298 and 20 K. Note that in the low-temperature spectra the 450 nm transition is clearly resolved in the two liganded enzyme forms (compare these spectra to the room-temperature second-derivative spectra shown in Figs. 4 and 5). These data are in good agreement with earlier 77 K difference spectra of reduced CO and CN inhibited mitochondria obtained by Wilson and Gilmore (1967), and confirm that the 450 nm band is indeed a genuine component of the parent absorption spectra of these enzyme forms. Importantly, these data indicate that the 450 nm band contributes to the room-temperature absorption spectra of cytochrome *c* oxidase, but is not well resolved.

THE 450 nm BAND IS SENSITIVE TO pH AND CYTOCHROME *c* BINDING

If the 450 nm transition reflects a unique structure of the cytochrome *a* binding pocket, one might expect that the relative intensity of this spectral feature might be sensitive to conditions that affect other physicochemical characteristics of cytochrome *a*. For example, it has been known for some time that the redox potential of cytochrome *a* is pH dependent in the CN-inhibited form of the enzyme, but much less so for the unliganded or CO-bound enzyme (Wikström *et al.*, 1981). We therefore studied the pH dependence of the second-derivative spectra of the fully reduced, fully reduced CO-bound, fully reduced CN-bound, and CN-inhibited mixed-valence enzyme (Ishibe *et al.*, 1991). We found no significant effect of pH on the spectra of the unliganded or CO-bound forms. However, both the mixed valence and fully reduced CN-inhibited enzyme forms showed a dramatic pH dependence. This is illustrated for the mixed-valence enzyme in Fig. 7. As the pH is lowered, there is a dramatic diminution of intensity for the 450 nm band, which is completely reversible if one readjusts the pH to 7.4. Figure 7 also plots the relative intensity ratio 450/444 nm as a function of pH for this enzyme form. The solid line through the data is the nonlinear least-squares best fit to the Henderson–Hassalbalch equation for a single titratable group, and provides an estimate of the transition pK_a of 6.6 $\pm$ 0.1. Interestingly, this pK_a value is identical, within experimental error, to that obtained for the pH dependence of the overall rate of enzyme turnover and to that predicted for the transition between proton input and output states in the proton pumping model of Malmström (1986).

It is also believed that complex formation between cytochrome *c* and cytochrome *c* oxidase can

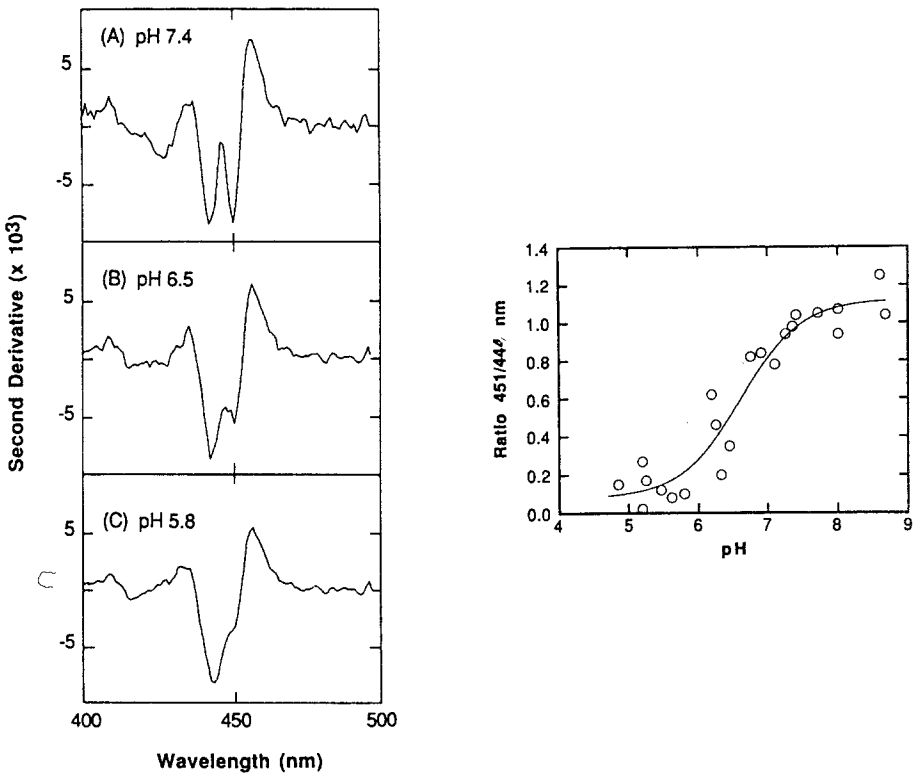

Fig. 7. (Left) Effects of pH on the second-derivative absorption spectrum of CN-bound mixed-valence bovine cytochrome *c* oxidase. (Right) Plot of the ratio of 451/444 nm intensity in the second-derivative spectra of the CN-bound mixed-valence bovine enzyme as a function of pH. The solid line through the data represents the least-squares best fit of the data to the Henderson–Hasselbalch equation for a single titratable group. Data taken from Ishibe *et al.* (1991).

affect the conformation of cytochrome *a*. We have therefore studied the 1 : 1 complexes between cytochrome *c* and various stable forms of the enzymes from bovine heart and *Paracoccus denitrificans* (Lynch *et al.*, 1992; Lynch and Copeland, 1992). For both species we find that complex formation between ferrocytochrome *c* and the cyanide-inhibited mixed-valence or fully reduced enzyme collapses the two-band pattern seen in the second-derivative spectra (i.e., 443 and 450 nm bands) to a single band at ca. 444 nm. Disruption of the electrostatic complex between these two proteins by increased ionic strength completely reverses this spectral perturbation, suggesting that these changes are associated with complex formation. Interestingly, these spectral changes are not observed for the reduced CO-bound enzyme, nor are they mimicked by complex formation between the enzyme and poly-L-lysine. These changes in second-derivative spectra are correlated with similar changes in the Soret region circular dichroic (CD) spectra of these complexes (Lynch and Copeland, 1992). Thus perturbations of the cytochrome *a* electronic transitions accompany complex formation with ferrocytochrome *c*. These perturbations could be due to changes in ground-state structure of the heme cofactor, or could reflect changes in the surrounding polypeptide that affect the energy of the ground or excited electronic states of the heme. To address this issue further, we studied the resonance Raman spectra of these complexes under conditions where the cytochrome *c* oxidase hemes are selectively enhanced (Lynch and Copeland, 1992). No significant changes in cytochrome *a* vibrational modes were observed between the intact complexes and the same samples after disruption of the complexes with high salt. Since the vibrational bands in the resonance Raman spectra reflect the electronic ground-state structure of the heme, we concluded that the perturbations observed in the second-derivative and CD spectra most likely reflect changes in the surrounding polypeptide that affect the energy of the π^* state of the heme. We extend this conclusion to suggest that the observation

of a 450 nm band in the second-derivative spectra of any oxidase form is dependent on specific protein–chromophore interactions that are unique to the cytochrome *a* binding pocket of the native enzyme.

THE 450 nm BAND IS UNIQUE TO NATIVE CYTOCHROME *a*

We have speculated above that the observation of the 450 nm band is dependent on specific protein–chromophore interactions that are unique to the cytochrome *a* binding pocket of native cytochrome *c* oxidase. If, for example, the 443 and 450 nm bands represent two distinct conformations of the surrounding polypeptide, the longer-wavelength 450 nm species might be the result of the movement of a charged or aromatic amino acid into closer proximity to cytochrome *a*, providing a basis for perturbation of the π system of the heme.

To test the universality of this 450 nm transition, we have first surveyed the second-derivative spectra of cytochrome *c* oxidases from a number of species. To date we have observed the 450 nm band in liganded forms of the cytochrome *c* oxidases from bovine heart, rat liver, *Paracoccus denitrificans*, *Rhodobacter sphaeroides*, the *aco* oxidase from *Bacillus* YN-2000, and the aa_3-600 ubiquinol oxidase from *Bacillus subtilis*. This last enzyme is unique in that it contains the two heme A cofactors of the cytochrome *c* oxidases, but lacks the Cu_A center; but its second-derivative spectra are nevertheless similar to the other cytochrome *c* oxidases. Additionally, Ferguson-Miller and coworkers have observed the 450 nm band in the second-derivative spectra of oxidases from higher plants, such as wheat and maize (S. Ferguson-Miller, personal communication). Thus, as far as we have been able to test, the 450 nm band appears to be a universal feature of terminal oxidases that contain the cytochrome *a* cofactor. In contrast to these results, in collaboration with Dr. James Fee (Los Alamos National Laboratories), we have found that cyanide binding to the reduced form of the cytochrome ba_3 of *Thermus thermophilus* does not lead to the appearance of a 450 nm feature in the second-derivative spectrum (Horvath *et al.*, unpublished data).

We next studied the effects of limited protein denaturation on the second-derivative spectra of the bovine enzyme. Using either thermal or chemical (urea or guanidine hydrochloride) denaturation, we found that disruption of the native structure of the

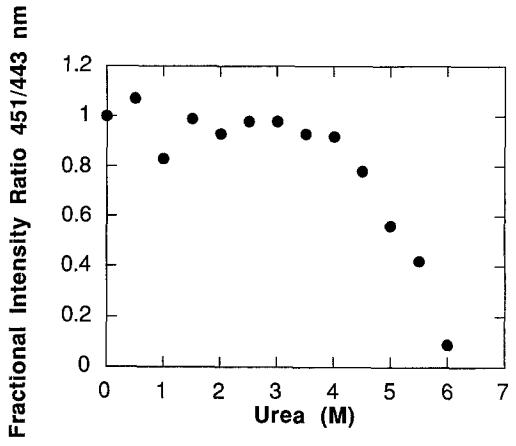

Fig. 8. Effect of urea on the 451/443 nm intensity ratio for the second-derivative spectrum of the CN-bound mixed-valence bovine enzyme.

cyanide-bound mixed-valence enzyme led to a sharp diminution of the 450 nm band. For example, in Fig. 8 we illustrate the lost of 450 nm band intensity that occurs with increasing levels of urea for the cyanide-inhibited mixed-valence bovine enzyme. Thus, disruption of the native conformation of the enzyme leads to the selective loss of the 450 nm band in the second-derivative spectrum, supporting our hypothesis of specific protein–chromophore interactions leading to this absorption feature (Perry *et al.*, unpublished data).

We have also attempted to prepare other imidazole-based 6-coordinate low-spin heme A species to test whether the 450 nm band is a common feature of these compounds, or is unique to the environment within the enzyme. We have prepared monodispersed ferrous heme A bis-imidazole in 1% sodium dodecyl sulfate (SDS) and found that this species gives rise to only a single Soret transition at ca. 440 nm in its second-derivative spectrum. Likewise, in collaboration with Professor William T. Morgan (University of Missouri), we have bound heme A to rabbit serum histidine-rich glycoprotein (HRG) to produce a bis-histidine-liganded low-spin heme. As illustrated in Fig.9, the absorption spectra of this heme A–protein complex is remarkably similar to those of cytochrome *a*. Despite the similarity in parent absorption spectra, however, the second-derivative spectrum of ferrous heme A–HRG shows no evidence of a 450 nm absorption feature. Similarly, in collaboration with Professor Masao Ikeda-Saito (Case Western Reserve University), we have reconstituted heme A into a mutant of recombinant human myoglobin, in which His64 is replaced by a Val residue and Val68 is replaced by a

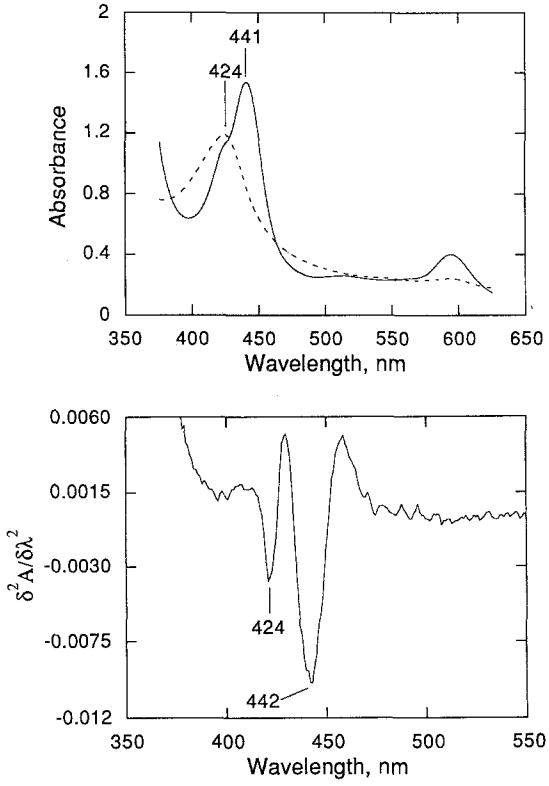

Fig. 9. (Top) Absorption spectra of oxidized (dashed line) and dithionite-reduced (solid line) heme A-reconstituted rabbit serum histidine-rich glycoprotein. (Bottom) Second-derivative spectrum of the reduced form of heme A-reconstituted rabbit serum histidine-rich glycoprotein. The band at 424 nm is due to residual oxidized heme.

His. As with other hemes, this mutant myoglobin forms a bis-histidine-liganded, low-spin species when reconstituted with heme A. Again, the second-derivative absorption spectrum of the reduced form of this complex reveals a single heme A Soret transition at 438 nm and no evidence of a 450 nm feature. Taken together, these results suggest that the 450 nm-absorbing species is restricted to ferrous cytochrome *a* within the binding pocket of the native enzyme (Felsch *et al.*, unpublished data).

FUTURE PROSPECTS

As reviewed here, our laboratory has shown clear evidence for a second Soret transition of ferrous cytochrome *a* that appears to be intensified in ligand-bound forms of the enzyme. We have speculated that this second transition, at 450 nm, may represent an alternative conformation of the cytochrome *a* binding pocket. The ability to access two distinct conformations for this cofactor could be an important aspect of the catalytic mechanism of cytochrome *c* oxidase. The specific role of such conformational switching in various aspects of oxidase function remains to be elucidated. As stated earlier, the appearance of two Soret transitions may be a general feature of the native conformation of the cytochrome *a* binding pocket of the enzyme, and not the result of conformational heterogeneity. If this latter explanation is correct, it indicates an extremely unusual environment for the heme cofactor in the enzyme. The high conservation of the 450 nm feature of cytochrome *a* across phyla and kingdoms would seem to identify in this case a special mechanistic significance for such a specialized heme-binding pocket. In part the resolution of these issues will depend on a better understanding of the structural determinants for this unique absorption feature of the enzyme.

What type of protein–chromophore interactions might be responsible for the observation of the 450 nm band? It is well known that red shifts of π–π^* transitions can be affected by increases in solvent polarity that selectively stabilize the π^* state of the molecule (Cantor and Schimmel, 1980). For a protein-imbedded chromophore, one must view the surrounding polypeptide as the solvent system. Charged amino acid side chains in the vicinity of the heme group could have strong perturbing influences on the local dielectric, and hence the spectral properties of the heme. If the 443 and 450 nm bands represent two conformational variants of cytochrome *a*, the structural difference between these two conformers could be the relative motion of one or a few charged amino acid residues. In other protein–chromophore systems, such as the chlorophylls, bacteriochlorophylls, and the visual pigments, point charge effects have been suggested as the determinants of the large red shifts observed in the absorption spectra of these systems (Davis *et al.*, 1981; Eccles and Honig, 1983; Kakitani *et al.*, 1985).

Alternatively, it has recently been shown that the red shifts associated with some photosynthetic pigments are, in part, due to π–π interactions between the chromophore and nearby aromatic amino acids (Fowler *et al.*, 1992). When these aromatic residues are replaced by site-directed mutagenesis, the lowest-energy π–π^* transition of the chromophore was blue shifted by as much as 24 nm. By analogy, the 450 nm band observed in cytochrome *c* oxidase might be the

result of strong cytochrome *a*-aromatic amino acid interactions.

Testing these possible explanations for the observed Soret transitions of cytochrome *a* will require the application of molecular biological methods to the oxidase superfamily. Fortunately, Gennis and coworkers have recently succeeded in cloning and expressing wild type and point mutants of the cytochrome *c* oxidase from *Rhodobacter sphaeroides*. With these mutant enzymes available, we can now begin to explore systematically the nature of protein–chromophore interactions within this enzyme. In collaboration with Professor Robert B. Gennis (University of Illinois), we have initiated a program aimed at answering some of these important issues in terminal oxidase structure and function.

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