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Probing protein-cofactor interactions in the terminal oxidases by second derivative spectroscopy: Study of bacterial enzymes with cofactor substitutions and heme A model compounds

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Abstract

Second derivative absorption spectra are reported for the aa_3-cytochrome *c* oxidase from bovine cardiac mitochondria, the aa_3-600 ubiquinol oxidase from *Bacillus subtilis*, the ba_3-cytochrome *c* oxidase from *Thermus thermophilis*, and the *aco*-cytochrome *c* oxidase from *Bacillus* YN-2000. Together these enzymes provide a range of cofactor combinations that allow us to unequivocally identify the origin of the 450-nm absorption band of the terminal oxidases as the 6-coordinate low-spin heme, cytochrome *a*. The spectrum of the *aco*-cytochrome *c* oxidase further establishes that the split Soret band of cytochrome *a*, with features at 443 and 450 nm, is common to all forms of the enzyme containing ferrocytochrome *a* and does not depend on ligand occupancy at the other heme cofactor as previously suggested. To test the universality of this Soret band splitting for 6-coordinate low-spin heme A systems, we have reconstituted purified heme A with the apo forms of the heme binding proteins, hemopexin, histidine-proline-rich glycoprotein and the H64V/V68H double mutant of human myoglobin. All 3 proteins bound the heme A as a (bis)histidine complex, as judged by optical and resonance Raman spectroscopy. In the ferroheme A forms, none of these proteins displayed evidence of Soret band splitting. Heme A-(bis)imidazole in aqueous detergent solution likewise failed to display Soret band splitting. When the cyanide-inhibited mixed-valence form of the bovine enzyme was partially denatured by chemical or thermal means, the split Soret transition of cytochrome *a* collapsed into a single band at 443 nm. Taken together these data suggest that the observation of Soret splitting, including a feature at 450 nm, results from specific protein-cofactor interactions that are unique to the cytochrome *a*-binding pocket of the terminal oxidases. The conservation of this unique binding pocket among evolutionarily distant species may reflect some mechanistic significance for this structure.

Keywords: cytochrome *c* oxidase; electron transfer; heme A; spectroscopy

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The terminal oxidases of aerobic respiration form a superfamily of structurally related enzymes that catalyze the reduction of molecular oxygen to water. During the course of intramolecular electron transfer within these enzymes, energy is utilized for the translocation of protons across the respiratory membranes. These translocated protons ultimately provide the thermody-

namic driving force for ATP synthesis within the respiratory membrane by another family of integral membrane enzymes, the ATP synthases (Nicholls, 1982). The terminal oxidases all contain 2 heme groups and at least 1 copper ion that function as mechanistically essential redox cofactors. One heme and 1 copper together form a binuclear site for oxygen binding and reduction. The other heme is always coordinated by a pair of histidine residues (i.e., it is always 6-coordinate low-spin) and serves as the requisite electron conduit to the binuclear ligand binding site (Saraste, 1990). The most well-studied group of enzymes in this superfamily are the mitochondrial cytochrome *c* oxidases. In these enzymes both heme groups are composed of protein-bound heme A chromophores. The oxygen-binding heme in these enzymes is referred to as cytochrome a_3, and the other (electron transfer) heme is referred to as cytochrome *a*. These enzymes additionally contain a second copper ion, Cu_A, within subunit II that serves as the initial electron acceptor for the physiological electron donor to the enzyme, ferrocytochrome *c* (Saraste, 1990).

Our group has been interested in following local structural transitions around the metal cofactors in these enzymes that attend enzymatic activity. Toward this end, we have introduced the use of second derivative absorption spectroscopy as a means of resolving and studying the individual electronic transitions of the 2 heme groups within the protein (Copeland, 1991, 1993; Ishibe et al., 1991; Sherman et al., 1991; Lynch & Copeland, 1992; Lynch et al., 1992). During the course of these studies we have discovered a new electronic transition of one of the hemes at ca. 450 nm. The molecular origins of this spectral feature are not fully understood at present. We have suggested that the 450-nm band is associated exclusively with the reduced form of cytochrome *a*, and that observation of this signal is dependent on specific protein–cofactor interactions that take place within the heme binding site of the native enzyme (Copeland, 1993). To test these hypotheses, and to gain further insight into the structural determinants of this new spectroscopic feature, we have undertaken a systematic study of the spectroscopic profiles of members of the terminal oxidase superfamily of enzymes and of related heme A-containing model systems. In this paper we report the results of optical spectroscopic studies for some of these systems. The results presented here indicate that the 450-nm band is indeed a unique feature of cytochrome *a* within the specialized heme binding pocket of the terminal oxidases.

Results

Spectra of terminal oxidases

When no ligands are bound at the cytochrome a_3 site, the second derivative spectra of reduced aa_3 oxidases display a single band at ca. 443 nm, with a weak shoulder at ca. 450 nm for ferrous cytochrome *a* and cytochrome a_3. However, when ligands, such as carbon monoxide, are bound to cytochrome a_3, the electronic transition for this heme is blue-shifted, unmasking the transitions for the low-spin heme, cytochrome *a*. In such forms of the enzyme one observes a doublet for the cytochrome *a* Soret transition, with peak centers at 443 and 450 nm. This is illustrated in Figure 1 for bovine cytochrome *c* oxidase. Extensive data for bovine and other cytochrome *c* oxidases have been previously reported (for a review, see Copeland, 1993). In all cases one observes the double Soret transition in forms of the enzyme

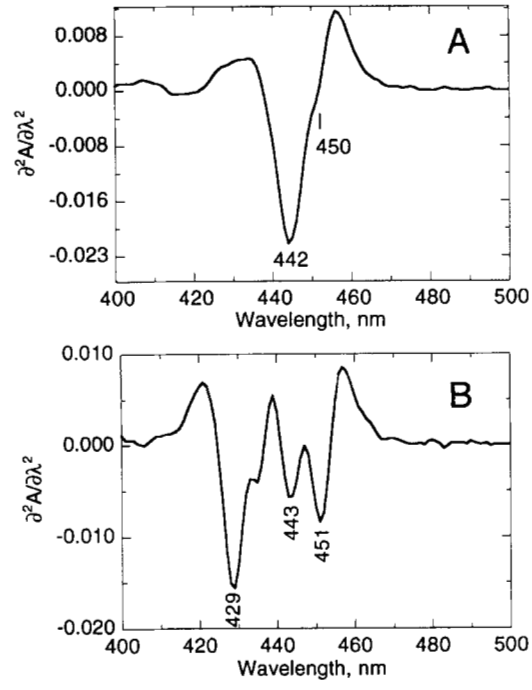

Fig. 1. Second derivative absorption spectra for bovine cytochrome *c* oxidase in the fully reduced (**A**) and reduced, carbon monoxide-bound (**B**) forms.

where cytochrome *a* is reduced and cytochrome a_3 is ligand bound. We have proposed that the 450-nm transition is associated exclusively with ferrocytochrome *a*. To test this hypothesis we have studied the second derivative spectra of 2 bacterial variants of the cytochrome *c* oxidase family of enzymes, the cytochrome ba_3 of *Thermus thermophilis*, and the cytochrome *aco* of *Bacillus* YN-2000. Cytochrome ba_3 is a cytochrome *c* oxidase in which the low-spin cytochrome *a* cofactor is replaced by a *b*-type heme so that the only heme A chromophore in this enzyme is cytochrome a_3. If our suggestion that the 450-nm band is exclusively associated with cytochrome *a* is correct, then this band should not be observed for the *T. thermophilis* enzyme. Figure 2 illustrates the absorption and second derivative spectra of this enzyme. Here the electronic transitions of the 2 hemes are well separated, and as anticipated, no evidence of an electronic transition at 450 nm is observed for this enzyme. It is also interesting to note that no evidence of Soret splitting is observed for the low-spin *b*-type heme in this enzyme, at least with the spectral resolution used here; thus, the split Soret feature appears to be a feature of cytochrome *a* only.

Figure 3 depicts the absorption and second derivative spectra of the cytochrome *aco* enzyme from *Bacillus* YN-2000. This enzyme has the cytochrome a_3 cofactor replaced by a heme *o* chromophore and also has a cytochrome *c* moiety covalently attached. In this enzyme the only heme A chromophore present is cytochrome *a*. The second derivative spectrum for this enzyme in its reduced form shows excellent separation of the electronic transitions of the 3 hemes. The cytochrome *a* Soret transition shows clear evidence of a doublet at 443 and 450 nm. Interestingly, this splitting of the cytochrome *a* transition is observed in the absence of exogenous ligands for this enzyme. These data

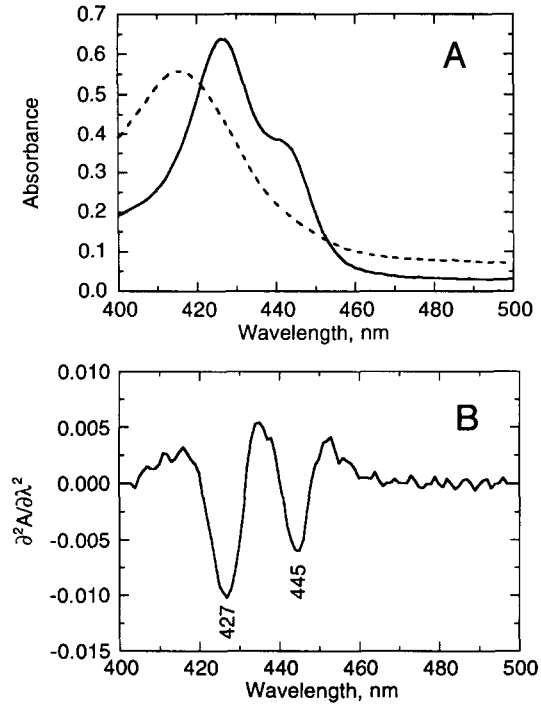

Fig. 2. A: Absorption spectra of resting (dashed line) and reduced (solid line) cytochrome ba_3 from *T. thermophilus*. **B:** Second derivative spectrum of fully reduced cytochrome ba_3 from *T. thermophilus*.

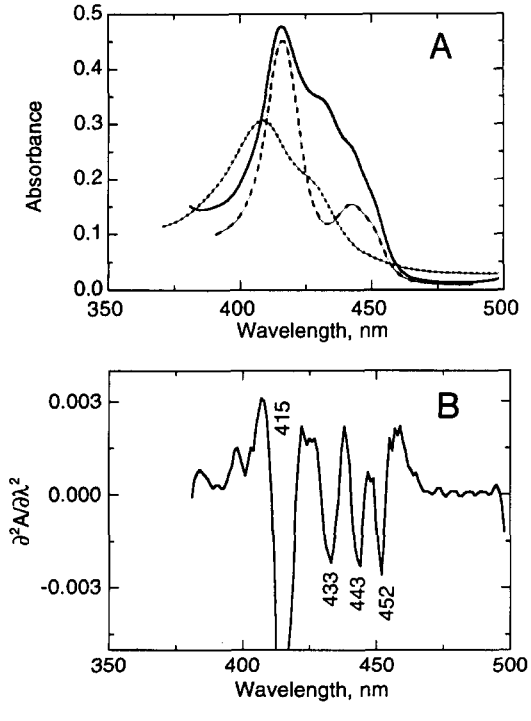

Fig. 3. A: Absorption spectra of resting (dotted line), reduced (solid line), and carbon monoxide-inhibited (dashed line) cytochrome *aco* from *Bacillus* YN-2000. **B:** Second derivative spectrum of fully reduced cytochrome *aco* from *Bacillus* YN-2000. The cytochrome *c* Soret transition (415 nm) is cut off to highlight the heme A transitions.

suggest that the Soret transition of ferrocytochrome *a* is always split in the oxidases, regardless of the ligand occupancy at cytochrome a_3. Our previous observation (Sherman et al., 1991) that the 450-nm band is seen only for liganded forms of the mammalian enzyme most likely reflects spectral overlap between the ferrocytochrome *a* and a_3 transitions that obscure the true intensity of the 450-nm band in these species.

We have previously shown that the double Soret transition of ferrocytochrome *a* collapses to a single band at 443 nm when ferrocytochrome *c* is bound to the enzyme at the Cu_A site of subunit II (Lynch & Copeland, 1992; Lynch et al., 1992). Li et al. (1988) and Nilsson et al. (1988) have shown that mild heat treatment of the bovine enzyme selectively disrupts the structure around Cu_A. Therefore, we heat treated the bovine enzyme in this way and then formed the cyanide-inhibited mixed-valence species of this perturbed enzyme. The second derivative spectrum of this sample showed significant reduction in the relative intensity of the 450-nm transition relative to the 443-nm band (data not shown), leading us to wonder if the 450-nm transition might result from electronic interactions between cytochrome *a* and Cu_A. It is known that some interactions between these 2 metals occur, and that they are manifested in anticooperativity of their redox potentials (Ellis et al., 1986; Wang et al., 1986). To test this possibility we purified the aa_3-600 ubiquinol oxidase of *Bacillus subtilis*. This enzyme is unique among aa_3-containing enzymes in that it lacks the Cu_A cofactor and hence is not a cytochrome *c* oxidase. The second derivative spectra of the reduced and reduced, carbon monoxide-bound forms of this enzyme are displayed in Figure 4. The 450-nm band is clearly present in this enzyme. Thus, electronic interactions with Cu_A do not appear to play a significant role as determinants of the occurrence of the 450-nm transition of cytochrome *a*.

The data presented above suggest that the 450-nm band is exclusively associated with ferrocytochrome *a* within the enzymes of the terminal oxidase superfamily. Data from our laboratory and others (see Copeland, 1993, for a review) have demonstrated that this transition is conserved across all plant, animal, and bacterial phyla that have been tested. In contrast, no split transition with intensity at 450 nm has ever been reported for ferroheme A chromophores other than the cytochrome *a* cofactor within the oxidases. We wondered, therefore, whether this transition is a universal feature of 6-coordinate low-spin heme A or is the result of specific protein–cofactor interactions unique to the terminal oxidases. To investigate this further we studied the effects of urea-induced denaturation of the bovine enzyme in its cyanide-inhibited mixed-valence form. Figure 5 illustrates the effect of urea on the intensity ratio for the 450- and 443-nm features of the second derivative spectrum for this form of the enzyme. The relative intensity of each of these spectral features was estimated by measuring the integrated area under the band as described by Ishibe et al. (1991). We must caution, however, that the measured parameter may not be directly related to changes in the corresponding absorption feature. Changes in other portions of the spectrum could possibly account for the observed diminution of the 450-nm component in the second derivative spectrum. Here we simply demonstrate that the observed 450-nm band intensity is selectively lost as the protein structure surrounding cytochrome *a* is perturbed. These data are consistent with the idea that specific protein–cofactor interactions are required to produce the splitting of the heme A Soret transition associated with cytochrome *a*. A more complete study of

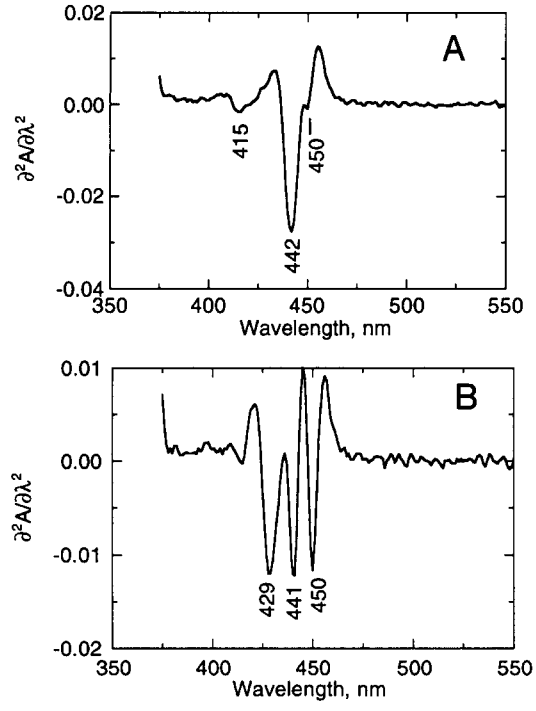

Fig. 4. Second derivative absorption spectra for *B. subtilis* aa_3-600 ubiquinol oxidase in the fully reduced (**A**) and reduced, carbon monoxide-bound (**B**) forms.

chemical-induced denaturation of the bovine and *Paracoccus denitrificans* enzymes will be reported separately (P.N. Goudreau, M.P. Horvath, & R.A. Copeland, manuscript in prep.).

Spectra of heme A model compounds

To test further the universality of the 450-nm transition, we prepared several 6-coordinate low-spin (bis) imidazole heme A model compounds and recorded their second derivative spectra. Figure 6 shows the absorption and second derivative spectrum of ferroheme A–(bis)imidazole in 1% (w/v) aqueous SDS. Babcock and coworkers have previously shown that, in this deter-

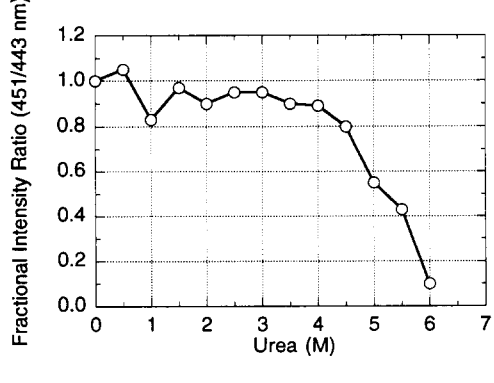

Fig. 5. Effect of urea on the 451/443-nm intensity ratio in the second derivative absorption spectrum of cyanide-inhibited mixed-valence bovine cytochrome *c* oxidase.

gent system, the heme A is monodisperse and present in the 6-coordinate low-spin form (Babcock et al., 1979). We have confirmed by resonance Raman spectroscopy that the compound in our hands is indeed 6-coordinate and low-spin (data not shown). For all of the model compounds studied here, reduction of the heme iron by sodium dithionite was incomplete for reasons that are not entirely clear. Thus, in the spectra presented here, one typically observes some Soret band intensity at ca. 420 nm due to some residual oxidized heme. The presence of this residual oxidized heme does not interfere with interpretation of the spectral data for the reduced heme form because the spectral signals for oxidized and reduced heme A are well separated. The spectra illustrated in Figure 6 clearly indicate that no transition at 450 nm is associated with ferroheme A–(bis)imidazole in detergent solution. At lower detergent concentrations heme A aggregates into multimeric structures (Vandekooi & Stotz, 1966; Choi et al., 1983) while remaining 6-coordinate low-spin (J. Felsch, unpubl. data). In this aggregated state the Soret transition shifts to ca. 456 nm. We have confirmed these results by obtaining spectra of ferroheme A–(bis)imidazole in 0.1% aqueous SDS (Fig. 7). Although the Soret transition does indeed shift to 456 nm under these conditions, it remains a single band with no evidence of splitting seen in the second derivative spectrum.

We have further prepared 6-coordinate low-spin heme A compounds using histidine residues within globular proteins as the fifth and sixth ligands to the heme. For this purpose we used 3 proteins known to bind hemes generally in 6-coordinate low-spin (bis)histidine fashion: rabbit serum histidine–proline-rich glycoprotein (HPRG); rabbit serum hemopexin (HPX); and a double point mutant, H64V/V68H, of human myoglobin (Qin et al.,

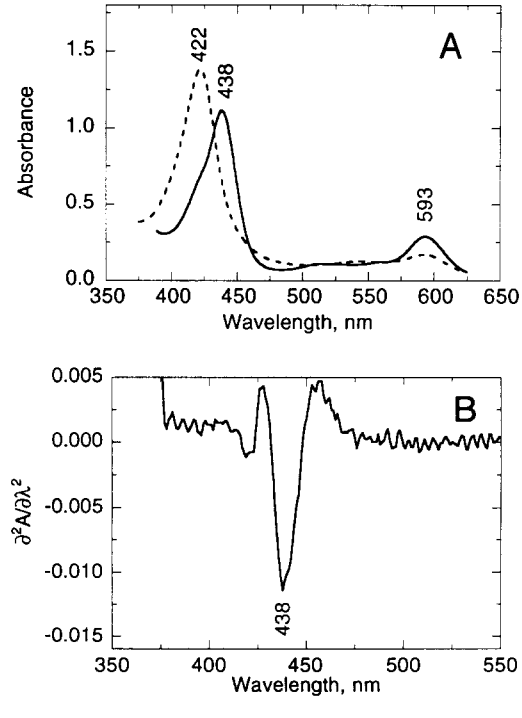

Fig. 6. A: Absorption spectra of oxidized (dashed line) and reduced (solid line) heme A–(bis)imidazole complex (10 μM) in 1% aqueous SDS (w/v). **B:** Second derivative spectrum of the reduced heme A–(bis)imidazole in 1% SDS.

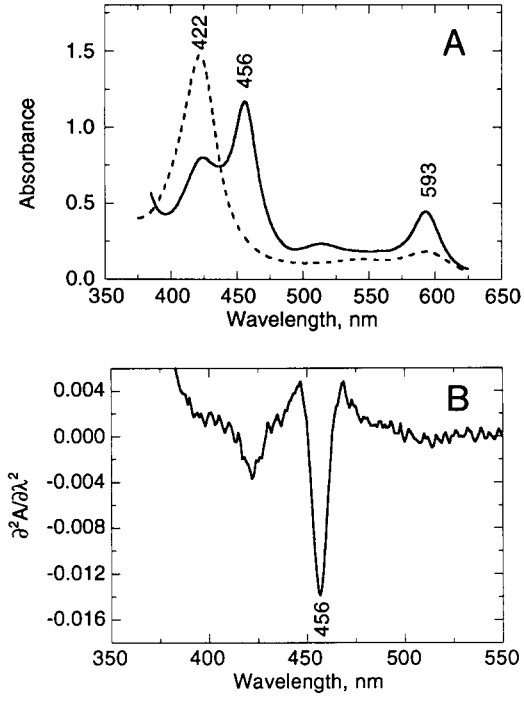

Fig. 7. A: Absorption spectra of oxidized (dashed line) and reduced (solid line) heme A–(bis)imidazole (10 μM) in 0.04% aqueous SDS (w/v). **B:** Second derivative spectrum of the reduced heme A–(bis)imidazole complex in 0.04% SDS.

1994). Reconstitution of these apoproteins with heme A and subsequent reduction of the heme led to 6-coordinate low-spin heme binding as assessed by optical and resonance Raman spectroscopy (data not shown). Figures 8 and 9 show the resulting absorption and second derivative spectra for the heme A-reconstituted forms of these heme binding proteins. In none of these proteins does one observe evidence of Soret band splitting in the reduced heme.

Discussion

The data presented here allow us to make several conclusions regarding the nature of the 450-nm electronic transition that is universally observed in reduced forms of cytochrome *c* oxidases. First, it is clear from comparison of the spectra of the various terminal oxidases that this transition is associated mainly with ferrocytochrome *a*; the other heme group of the enzyme does not appear to contribute significantly to this absorption band. Thus, no evidence of a transition at 450 nm is observed in the spectrum of the cytochrome ba_3 from *T. thermophilis*. Although these data do not allow us to rigorously conclude that there is no contribution to the 450-nm band from cytochrome a_3, any contribution from this chromophore must be very small.

Second, the spectrum of the cytochrome *aco* from *B. subtilis* clearly demonstrated that the 450-nm band is associated with ferrocytochrome *a* regardless of the ligation or valence state of the other heme cofactor. These data are inconsistent with our previous suggestion that the 450-nm band arises from conformational heterogeneity around cytochrome *a* that is induced by ligand binding at the other heme. Apparently, a special protein environment exists in cytochrome *a* that results in splitting of the Soret band for this heme, and this environment occurs in all forms of the enzyme. Conformational heterogeneity could, in principle, still account for the split Soret band of cytochrome *a*, but this heterogeneity would have to be present in all forms of the enzyme. Data from spectroelectrochemical titrations of the signals at 443 and 450 nm, which will be presented separately (M. Horvath, unpubl. data), indicate that the 2 signals arise from a common population. Thus, a more likely explanation for the present results is that specific protein-cofactor interactions occur within the binding pocket for cytochrome *a* that serve to lower the effective symmetry of the heme sufficiently to lift the degeneracy of the π^* molecular orbital associated with the Soret transition (Sherman et al., 1991).

Finally, the change in π^* state-degeneracy seems to require very specific interactions between the heme A and the surrounding protein. Partial denaturation of the enzyme by chemical or thermal means suffices to eliminate the observed band-splitting. Likewise, 3 distinct protein-based model systems employed in this study failed to produce the large splitting of the heme Soret band that is seen for cytochrome *a*. Two of these proteins, HPRG and HPX, are well known to bind a variety of hemes in a (bis)histidine fashion, producing a 6-coordinate low-spin species. The other model system is based on a double mutant of human myoglobin that was specifically designed to bind hemes via (bis)histidine coordination. In this mutant the distal histidine H64, which normally participates in hydrogen bonding to exogenous ligands, is replaced by a valine residue, and V68, 1 helical turn away, is replaced by a histidine residue. ^{1}H NMR studies showed that His at position 68 coordinates to the heme iron to form a 6-coordinate species that is predominantly in a low-spin state (Qin et al., 1994). Despite the fact that all these

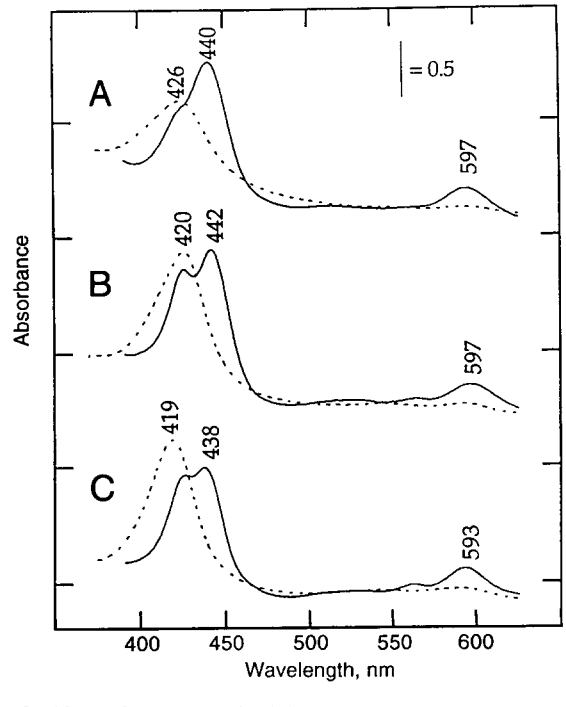

Fig. 8. Absorption spectra of oxidized (dashed lines) and reduced (solid lines) forms of heme A reconstituted into **(A)** rabbit serum histidine-proline rich glycoprotein, **(B)** rabbit serum hemopexin, and **(C)** recombinant H64V/V68H human myoglobin.

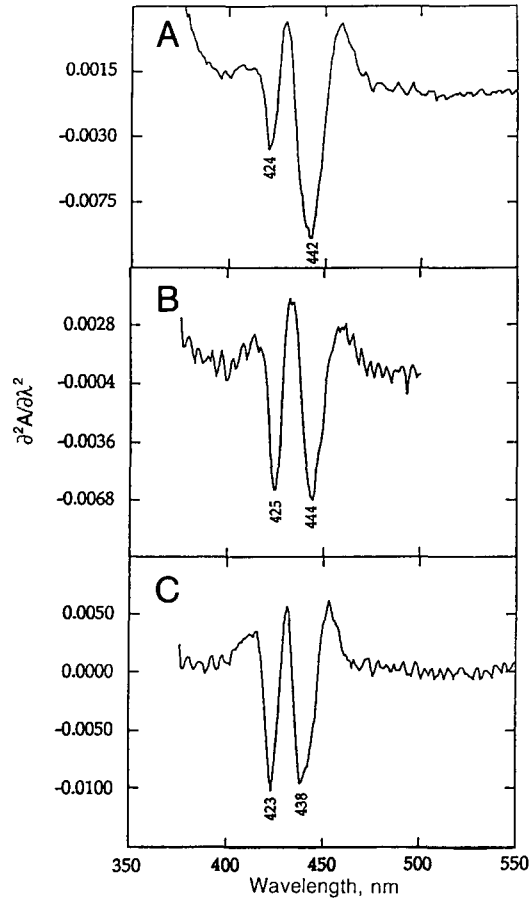

Fig. 9. Second derivative spectra of the reduced forms of heme A reconstituted into (**A**) rabbit serum histidine-proline rich glycoprotein, (**B**) rabbit serum hemopexin, and (**C**) recombinant H64V/V68H human myoglobin.

proteins successfully incorporated heme A in the expected (bis)histidine ligation form, none produced the Soret splitting exhibited by cytochrome *a*.

Our inability to reproduce this splitting in any small molecule or protein-based heme A model system speaks to the uniqueness of the structural determinants of this spectral feature. This singular environment is likely to be of mechanistic importance to this class of enzymes because the resulting spectral feature appears to be strictly conserved across evolutionarily distant species. What role such a specialized heme environment might play in the enzymatic activity of these proteins remains to be elucidated.

The specific structural features of the cytochrome *a* binding site of the enzyme that lead to this unique spectroscopic property also remain to be elucidated. We have speculated in the past that specific interactions between charged and/or aromatic amino acids and the heme cofactor could account for the long-wavelength feature at 450 nm (Copeland, 1993). However, the splitting of the Soret band that is seen exclusively in the native enzyme is more likely the result of lowering of the effective symmetry of the heme group. As we have previously noted (Sherman et al., 1991), this symmetry lowering could be effected in a number of ways, including hydrogen bond formation between the formyl group of cytochrome *a* and a hydrogen donor from the polypeptide. Although the present results do not allow us to specify the exact nature of the protein–cofactor interactions that lead to the observed spectroscopic features of cytochrome *a*, they do further exemplify the uniqueness of these interactions. With the recent application of site-directed mutagenesis to the oxidase superfamily of enzymes (Hosler et al., 1993), it may soon be possible to pinpoint the specific interactions within the native enzyme that lead to Soret band splitting for cytochrome *a*.

Materials and methods

Bovine cardiac cytochrome *c* oxidase (Hartzell & Beinert, 1974), *T. thermophilus* cytochrome ba_3 (Yoshida et al., 1984; Zimmerman et al., 1988), *Bacillus* YN-2000 cytochrome *aco* (Qureshi et al., 1990; Yumoto et al., 1993), rabbit serum HPX (Hrkal & Muller-Eberhard, 1971), and rabbit serum HPRG (Morgan, 1978) were purified as previously described. The aa_3-600 ubiquinol oxidase was purified by the method of Lauraeus et al. (1991) with minor modifications, from *B. subtilis* cells grown as described (Lauraeus et al., 1991). The absence of Cu_A in this enzyme was confirmed by measuring the near infrared absorption spectrum of this enzyme. Terminal oxidases containing Cu_A display a characteristic absorbance at ca. 830 nm (ϵ = 2 mM^{-1} cm^{-1}) that was lacking in this protein. Recombinant H64V/V68H human myoglobin was purified as previously described (Qin et al., 1994).

Heme A was purified from bovine cytochrome *c* oxidase as described by Takemori and King (1965). The purity of the isolated chromophore was assessed by TLC on silica gel IB2-F (J.T. Baker, Inc., Phillipsburg, New Jersey) in methylene chloride; all samples used in this work displayed a single spot on TLC with UV illumination. Heme A–(bis)imidazole complexes were prepared as described by Vandekooi and Stotz (1966) with the exception that the heme A was introduced into the aqueous imidazole solutions from a concentrated stock in dimethyl sulfoxide. Heme A concentration was determined by the pyridine hemochromogen method (Takemori & King, 1965).

Heme A was reconstituted with HPRG and HPX by addition of a concentrated stock of heme A in dimethyl sulfoxide to an aqueous solution (50 mM potassium phosphate, 10 mM NaCl, pH 7.4) of apoprotein to a final heme to protein ratio of 1.0:1.2 at room temperature. Protein concentrations were determined using extinction coefficients of 42.5 mM^{-1} cm^{-1} and 120 mM^{-1} cm^{-1} at 280 nm for HPRG and HPX, respectively. The axial coordination of the heme by the protein was monitored by the red shift of the heme Soret band from 415 to ca. 420 nm, which was complete within 30 min. Spectra of the reconstituted proteins were recorded within 8 h of preparation.

Recombinant H64V/V68H myoglobin was converted to the apoprotein by the 2-butanone method of Teale (1959) as modified by Yonetani (1967) with the exception that the butanone-extracted apoprotein was dialyzed first against 2 changes of distilled water followed by 2 changes of 10 mM Tris-HCl, pH 8.0. The apoprotein solution was then centrifuged at 35,000 × *g* for 30 min to remove any precipitated protein. Conversion to the apoprotein was >99% successful as assessed by the ratio of absorption at 280–409 nm (Yonetani, 1967). The apomyoglobin was flash-frozen with dry ice/ethanol and stored at −80 °C until use. Apomyoglobin was reconstituted with heme A as described above for HPRG and HPX. Axial coordination of the heme by the protein was monitored by the red-shift of the Soret

band from 415 to 419 nm. We found that the 6-coordinate species was only stable for ca. 6 h. After that time the protein converted to a 5-coordinate heme species. All of the reported spectra were obtained early after complete 6-coordinate heme-protein adducts were formed. For all of the model systems describe here, heme A was introduced from a concentrated stock solution in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in any of these solutions was <0.3% (v/v).

Optical absorption and second derivative spectra were recorded with a computer-interfaced Cary 14 UV-vis spectrophotometer as previously described (Sherman et al., 1991). All of the reported spectra were obtained with samples at a final heme concentration of 5–10 μM in a 1-cm-pathlength cuvette. Resonance Raman spectra of all samples were recorded with 441.6 nm excitation from a He-Cd laser as previously described (Ishibe et al., 1991).

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