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HEME *c* - HEME d_1 INTERACTION IN *PSEUDOMONAS* CYTOCHROME OXIDASE (NITRITE REDUCTASE): A REAPPRAISAL OF THE SPECTROSCOPIC EVIDENCE

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SUMMARY: Magnetic circular dichroism (MCD) spectra of *Pseudomonas aeruginosa* cytochrome oxidase are reported over the spectral range of 350-700 nm for the oxidized, ascorbate-reduced, dithionite-reduced and reduced carbon monoxide forms. The spectra of all forms examined can be interpreted as the simple sum of the individual heme *c* and heme d_1 contributions without invoking "heme-heme interaction." In particular and contrary to a recent report [Orii, Shimada, Nozawa, and Hatano, this Journal 76, 983 (1977)] no effect of ligand binding to ferrous heme d_1 was observed in the MCD spectrum of the heme *c* component. It seems likely that the previous findings were the result of incomplete reduction of the enzyme in the absence of stabilizing ligands.

Pseudomonas cytochrome oxidase (ferrocytochrome c_{551}:oxidoreductase, EC 1.9.3.2) is the terminal respiratory enzyme of *Pseudomonas aeruginosa* (1,2) and can transfer electrons to molecular oxygen or to nitrite, the latter being the probable physiological acceptor (3). The enzyme exists as a dimer of two equivalent subunits, each of which contains one heme *c* and one heme d_1 (4); thus the enzyme is capable of storing and discharging four electrons and has served as a model for comparison with mammalian cytochrome *c* oxidase. While a variety of experimental approaches have provided evidence of interactions between the heme and copper centers in the mammalian enzyme, no indications of heme *c*-d_1 interactions had been observed in the bacterial enzyme. Recently, however, a preliminary report has appeared suggesting that some form of interaction, manifest in the magnetic circular dichroism (MCD) spectra, exists in the reduced state of the *Pseudomonas* enzyme (5). These findings are in direct contrast to some of our unpublished experi-

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ments using the same methods and are surprising in view of the relative insensitivity of the MCD of low spin ferrocytochromes to subtle changes in the heme electronic structure (see 6 and references therein). Therefore we thought it important to communicate our results to date and to offer an explanation for the apparent interaction recently reported.

MATERIALS AND METHODS

Pseudomonas cytochrome oxidase was purified as previously described (7). The concentration of the enzyme, expressed on the basis of a monomer containing heme *c* and d_1, was determined using $\varepsilon_{411} = 148$ and $\varepsilon_{417} = 189$ $(\mathrm{mM{\cdot}cm})^{-1}$ for the oxidized and reduced samples, respectively. Sodium dithionite (Virginia Smelting) and ascorbic acid (Fisher) were added as solids, both in minimal amounts for reduction and in large excess; the only difference noted was the shift in the low energy heme d_1 band at high concentrations of dithionite as described by Parr *et al.* (8).

Absorption spectra were recorded using a Cary 14 spectrophotometer. MCD spectra were obtained with the instrument described by Sutherland *et al.* (9) using a bandpass of 1.2 nm. Two scans for each field direction were used to correct for natural CD and for signal averaging; multiple scans also provided a check against time-dependent changes in the spectra. Absorption spectra were recorded before and after the MCD spectra especially to insure that no auto-oxidation of reduced samples had occurred. The MCD data are presented as $\Delta\varepsilon/H$ $(\mathrm{M{\cdot}cm{\cdot}Tesla})^{-1}$ where one Tesla = 10^4 Gauss and can be converted to units of $[\theta]_m$ $(\mathrm{deg{\cdot}cm^2/decimole{\cdot}Gauss})$ by dividing with a factor of 3.03.

RESULTS AND DISCUSSION

The absorption and MCD spectra of the oxidized, ascorbate-reduced, and ascorbate-reduced carbon monoxide complex of the enzyme are shown in Fig. 1A.

In the resting, oxidized enzyme an intense derivative-shaped MCD curve typical of low spin (S = 1/2) ferric heme complexes of D_{4h} symmetry (6,10) is observed in the near UV region. The shape resembles the derivative of the Soret transition with extrema at 404 and 417 nm and a zero-crossing at 411 nm, the peak position of the Soret absorption band. The intensity is almost identical to that of mammalian mitochondrial cytochrome *c* (6) indicating similar paramagnetic ground states. Little MCD intensity, however, is observed in the 420-440 region where a broad absorption transition presumed due to ferric heme d_1 occurs. In the visible region the oxidized enzyme exhibits a weak S-shaped band centered about 559 nm which probably corresponds to the Faraday *A* term expected for the Q_0, or α band of ferric heme *c*. The

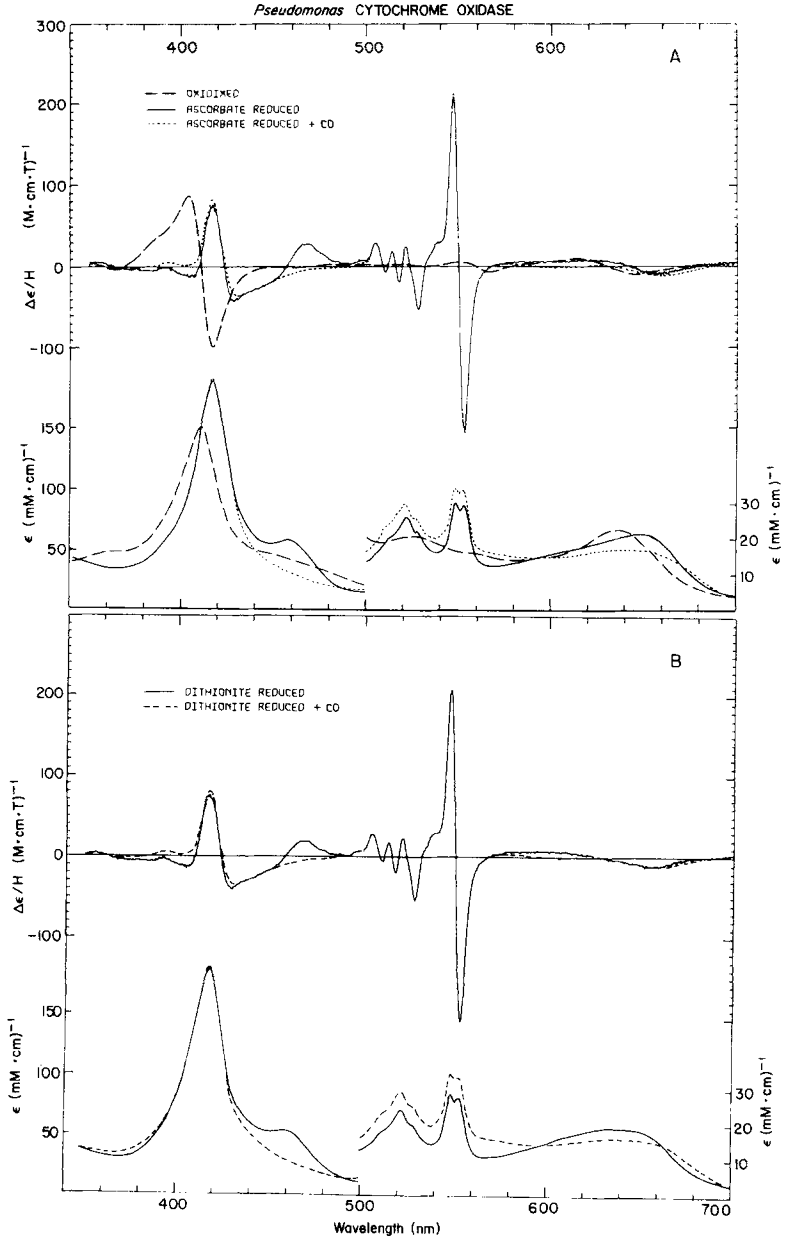

Figure 1. Absorption and MCD spectra of *P. aeruginosa* cytochrome oxidase in 0.1 M potassium phosphate, pH 7.0, 10°C. In A, the oxidized enzyme (— —) was reduced with an excess of ascorbic acid (———) and then placed under an atmosphere of carbon monoxide (-----). In B, the enzyme was reduced with a slight excess of sodium dithioniate (———) and then placed under an atmosphere of carbon monoxide (-----).

peak at 614 nm and the trough at 648 nm are due to Faraday β terms which arise from the x and y polarized Q bands of heme d_1.

When the enzyme is reduced with ascorbate, the spectra change dramatically. Starting from aerobic conditions the changes require several minutes to reach completion but then appear to be stable for at least several hours in the absence of oxygen. The MCD feature with extrema at 417 and 430 nm is very similar to that reported for mitochondrial ferrocytochrome *c* (6) and is consistent with a low spin, $S=0$, state for the reduced heme *c*. This similarity also extends to the visible region where a sharp A term with a peak of 547 nm, a zero-crossing at 550 nm, and a trough at 553 nm is associated with the heme *c* Q_0 band. The MCD intensity of this band is somewhat weaker than that of mitochondrial cytochrome *c* due to the increased bandwidth of the *Pseudomonas* hemochrome (the increased bandwidth is clearly evidenced in the splitting of the absorption spectrum). In the reduced state heme d_1 also exhibits a prominent Soret MCD peak near 467 nm, just to low energy of the absorption maximum which occurs at 460 nm. The visible bands of heme d_1 are also slightly red-shifted upon reduction.

The binding of carbon monoxide to the ascorbate-reduced enzyme alters the signals attributed to heme d_1 but not those of heme *c*. The heme d_1 Soret absorption and MCD signals near 460 nm disappear and the bands in the 600-700 nm region are broadened. With respect to the heme *c* component only minor changes occur in the 400-440 nm region (these may reflect weak heme d_1 contributions), and the signal centered about 550 nm is increased in intensity by only one percent.[a]

These results are consistent with CO binding solely to and affecting only the spectroscopic properties of heme d_1 in the ascorbate-reduced enzyme. They provide no evidence for the properties of ferro-heme *c* moiety being sensitive to ligation, and presumably, spin state changes in ferro-heme d_1. Orii *et al.* (5) on the other hand did observe changes in the heme *c* signals

[a] The changes in the absorption intensity in the heme *c* Q_0 and Q_v bands are due to changes in the broad underlying heme d_1 spectrum on CO binding.

on the addition of CO and other ligands to dithionite-reduced samples. Our results with samples reduced with dithionite, however, are very similar to those obtained using absorbate.

Figure 1B shows that the MCD spectra below 600 nm of samples reduced with dithionite and treated with CO are almost indistinguishable from the ascorbate-treated enzyme[b]; only a small difference in the intensity of the visible bands of reduced heme d_1 exists between the ascorbate and dithionite samples and this difference is due presumably to oxy-sulfur anion binding (8). Both reduced samples exhibit the expected low spin ferro-heme *c* type of MCD spectrum in the presence or absence of CO. In contrast Orii *et al.* (5) reported that the ferro-heme *c* features only fully appear after the addition of CO. However, close examination of their data (see Figure 1, Reference 5) also reveals that the MCD feature at 467 nm due to ferro-heme d_1 cannot be detected in the reduced sample. Since we have never failed to observe this MCD peak in the reduced enzyme, we can only conclude that either their sample was not fully reduced or partial degradation of the heme d_1 component had occurred in their preparation. Unfortunately the authors did not present absorption spectra which would help to characterize the exact redox state of their preparation: from the intensity of the Q_0 band *A* term (see Figure 1, Reference 5) we estimate that the heme *c* was ca. 60-70% oxidized, and from the lack of the 467 nm MCD signal it appears that heme d_1 was completely oxidized. With this in mind, it seems likely that the striking effect of CO on the intensity of the ferro-heme *c* MCD signals reported by Orii *et al.* simply reflects increased reduction of the enzyme. Carbon monoxide is known both to inhibit the enzyme and to lead to its auto-reduction (11). The other treatments that Orii *et al.* found to increase the ferro-heme *c* MCD intensity, namely, alkaline pH, imidazole binding and heme d_1 removal, also inhibit the enzyme and would be expected to prevent its auto-oxidation. It is also note-

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[b] We have noted some variability in the intensity of the ferro-heme d_1 Soret absorption (460 nm) and MCD (467 nm) in the presence of dithionite and the signals shown in Figure 1B are slightly weaker than those of the ascorbate-reduced sample (Figure 1A).

worthy that Orii *et al.* found that cyanide, which like carbon monoxide can bind to ferrous heme to yield a low spin complex, but which also binds tightly to ferric heme, failed to elicit the changes seen with CO.

In conclusion the absorption and MCD properties of the oxidized, reduced and liganded-reduced states of *Pseudomonas* cytochrome oxidase can be explained without invoking a heme-heme interaction which alters the intrinsic optical properties of the individual components.

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