Evidence for Modulation of the Heme Absorptions of Cytochrome *c* Oxidase by Metal–Metal Interactions†

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ABSTRACT: Four different methods based upon ligand inhibition of cytochrome a_3 and Cu_B have been applied to the determination of the reduced minus oxidized difference spectrum of cytochrome *a* of cytochrome *c* oxidase. (1) Cyanide was used to stabilize cytochrome a_3 and Cu_B in their oxidized states while cytochrome *a* and Cu_A were reduced; the spectrum of this species was compared with that of the fully oxidized cyanide complex. (2) formate was used to stabilize cytochrome a_3 and Cu_B in their oxidized states while cytochrome *a* and Cu_A were reduced; the spectrum of this species was compared with that of the fully oxidized formate complex. (3) Nitric oxide was used to stabilize cytochrome a_3 and Cu_B in their reduced states while cytochrome *a* and Cu_A were oxidized; the spectrum of this species was compared with that of the fully reduced, NO-associated enzyme. (4) Nitric oxide and azide were used together to prepare an enzyme species in which cytochrome a_3 was reduced and associated with a molecule of NO while Cu_B, cytochrome *a*, and Cu_A remained oxidized; this species was then compared with the fully reduced, NO-associated enzyme. The four methods of determining the reduced minus oxidized difference spectrum of cytochrome *a* give different results, especially with respect to the intensity of the α band near 604 nm. These results indicate that interactions between the metal centers influence the heme absorptions of the enzyme. Comparison of methods 3 and 4 indicates that the redox state of Cu_B strongly influences the absorption properties of one or both of the cytochromes. On the basis of the proximity of cytochrome a_3 and Cu_B within the enzyme, it is suggested that the spectroscopic properties of cytochrome a_3 are primarily affected. The data further indicate that a weaker spectral interaction may take place between the "a sites" (cytochrome *a* and/or Cu_A) and the metals of the oxygen reduction site (cytochrome a_3 and Cu_B); comparison of the difference spectra suggests that this interaction also affects the absorption properties of cytochrome a_3 rather than cytochrome *a*. The implications of these results for the interpretation of electrochemical titrations are discussed briefly.

Cytochrome *c* oxidase, the terminal electron acceptor in the mitochondrial respiratory chain, catalyzes the reduction of dioxygen to water. The functioning enzyme contains four metal centers, two copper ions (Cu_A and Cu_B) and two irons in the form of heme *a* (Malmström, 1979). It has been established by a variety of methods (Babcock et al., 1976, 1981; Blumberg & Peisach, 1979) that one of the hemes (cytochrome a_3) is high spin, while the other (cytochrome *a*) is low spin, in both the fully oxidized and fully reduced states of the enzyme. Cytochrome a_3 binds exogenous ligands such as CN^-, N_3^-, CO, and NO; cytochrome *a* does not bind exogenous ligands (Malmström et al., 1979).

Cytochromes *a* and a_3 have strong visible and near-ultraviolet absorptions. The intensity of the α band at approximately 600 nm is substantially increased upon reduction of the enzyme; this absorbance change has frequently been monitored in potentiometric (Tiesjema et al., 1973; Leigh et al., 1974), reductive (Mackay et al., 1973; Babcock et al., 1978), and oxidative (Schroedl & Hartzell, 1977a,b) titrations. In order to interpret these experiments, it is important to know the extent to which each of the cytochromes contributes to the total reduced minus oxidized absorbance of the enzyme near 600 nm. This question has been addressed in numerous investigations (Keilin & Hartree, 1939; Yonetani, 1960; Horie & Morrison, 1963; Vanneste & Vanneste, 1965), but a consensus has not yet been reached.

In the earliest attempts to deconvolute the cytochrome oxidase absorption spectrum (Keilin & Hartree, 1939; Yonetani, 1960; Horie & Morrison, 1963), cyanide was used to stabilize cytochrome a_3 in the oxidized state while cytochrome *a* was reduced by dithionite or other reductants. The absorbance change accompanying this reduction was taken to represent the reduced minus oxidized difference spectrum of cytochrome *a*. Implicit in this approach is the assumption that the redox states of cytochrome *a* and Cu_A do not influence the extinction of the cytochrome a_3–CN complex and that the inhibition of cytochrome a_3 by cyanide is complete. If either of these assumptions does not hold, a substantial error in the estimated contribution of cytochrome *a* could result.

The early deconvolution experiments in which cyanide was used indicated that cytochrome *a* contributes between 72% and 80% to the total reduced minus oxidized absorbance at 604 nm and approximately 50% at 444 nm. This viewpoint, called the 80/20 hypothesis, was generally accepted until potentiometric (Tiesjema et al., 1973; Leigh et al., 1974) titrations suggested that each of the cytochromes contributes approximately 50% to the absorbance change at 604 nm (the 50/50 hypothesis). However, potentiometric titrations in the presence of CO (Wilson et al., 1972) appeared more consistent with the 80/20 hypothesis than with the 50/50 hypothesis. These potentiometric results form the basis of a model of cytochrome oxidase which incorporates the 80/20 hypothesis while postulating a strong interaction between the cytochromes which modulates their redox potentials (Wikström et al., 1976). Alternative models involving interactive cytochrome absorption properties have also been proposed (Wilson & Leigh, 1974). While some magnetic circular dichroism (MCD) data (Babcock et al., 1976) suggest that the Soret absorbance of the enzyme is reasonably well described by a noninteractive model of the cytochrome absorptions, the possibility of spectral in-

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teraction between the metal centers of the enzyme, particularly as manifested in the α band near 600 nm, has not been systematically investigated.

A variety of ligand complexes may, like the cyanide complex, by used to record the reduced minus oxidized difference spectrum of cytochrome *a*. These include high-spin (e.g., formate) complexes of cytochrome a_3^{3+} (Nicholls, 1976) as well as low-spin (e.g., CO and NO) complexes of cytochrome a_3^{2+} (Blokzijl-Homan & Van Gelder, 1971; Keilin & Hartree, 1939). Since these ligands exhibit a strong preference for either the reduced or the oxidized state, cytochrome a_3 is stabilized in one redox state in its ligand complexes; in most cases, Cu_B, which is located near cytochrome a_3 in the enzyme (Tweedle et al., 1978; Stevens et al., 1979), is also stabilized in one redox state by the associated ligand. Because the redox states of all of the metal centers are well-defined in these ligand complexes, it is possible to isolate spectral interactions from redox potential interactions. If the metal centers of the enzyme do not interact spectrally, all of the methods for recording the reduced minus oxidized difference spectrum of cytochrome *a* are expected to give the same result in spite of differences in the redox and/or ligation states of cytochrome a_3 and Cu_B. If spectral interaction between the metal centers does occur, the cytochrome *a* difference spectra obtained by the various methods should be dissimilar.

In order to resolve the question of the relative cytochrome contributions to the composite reduced minus oxidized absorbance of cytochrome oxidase near 600 nm and investigate the possibility of spectral interaction between the metal centers of the enzyme further, we have recorded reduced minus oxidized difference spectra of cytochrome *a* by using four different methods to stabilize the redox states of cytochrome a_3 and Cu_B. Cytochrome a_3 was stabilized in the oxidized, high-spin state by formate and in the oxidized, low-spin state by cyanide; in both of these complexes, Cu_B was also stabilized in the oxidized state. Cytochrome a_3 was stabilized in the reduced, low-spin state in two complexes with NO; in one complex, Cu_B was also reduced while in the other it remained oxidized.

The four spectral deconvolution methods give estimates of the reduced minus oxidized absorbance of cytochrome *a* at 604 nm which range from 13.7 to 17.4 mM^{-1} cm^{-1}. The estimates of the reduced minus oxidized absorbance at 444 nm are somewhat more consistent and range between 53 and 59 mM^{-1} cm^{-1}. The pronounced variation in the α band intensity of reduced minus oxidized cytochrome *a* is rationalized in terms of spectral interactions between the metal centers; it is proposed that the redox state of Cu_B and its position in relation to cytochrome a_3 strongly influence the absorption properties of this cytochrome. The implications of our results for the interpretation of optical data from electrochemical titrations are discussed.

Materials and Methods

Cytochrome *c* oxidase was isolated by the method of Hartzell & Beinert (1974). Heme *a* concentration was determined by using $\Delta\epsilon$ (reduced minus oxidized) = 24 mM^{-1} cm^{-1} at 605 nm (Van Gelder, 1966). The purified oxidase preparation contained 7 nmol of heme *a*/mg of protein. The enzyme was solubilized in 0.5% Tween-20–50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4. The concentrations used in the optical absorption experiments are given in the figure legends.

The cyanide derivative of oxidized cytochrome oxidase was prepared by the addition of solid KCN, to a final concentration of 100 mM, to the aerobic enzyme solution contained in an airtight cuvette (10-mm path length). Solid KH_2PO_4 was added simultaneously to a final concentration of 200 mM in order to produce the HCN which reacts with the oxidase. The resulting solution was incubated for 4 h or longer at 4 °C before optical spectra were recorded. The half-reduced cyanide complex was prepared by adding sodium ascorbate (6 mM) and 60 μM tetramethylphenylenediamine (TMPD) to the oxidized cyanide complex. Optical spectra were recorded after 30 min; these spectra did not differ significantly from those obtained after 16 h of incubation at 4 °C. EPR spectra of more concentrated samples of the half-reduced cyanide complex prepared in this way indicated that cytochrome *a* and Cu_A were completely reduced; the absence of a cyanocytochrome a_3 electron paramagnetic resonance (EPR) signal indicated that Cu_B remained oxidized. Johnson et al. (1981) have presented EPR data which indicate that Cu_B may be reduced by dithionite in the presence of cyanide and have further stated that Cu_B reduction is also observed when TMPD is used as the reductant. We have also observed Cu_B reduction by dithionite in the presence of cyanide as evidenced by the appearance of a cyanocytochrome EPR signal (unpublished experiments) but have not observed the corresponding effect within the typical incubation times of our optical and EPR experiments (<1 h) when ascorbate/TMPD is employed as reductant. The discrepancy between our results and those of Johnson et al. may be due to differences in cyanide concentration. We have employed 100 mM cyanide in the present experiments. Johnson et al. do not report the cyanide concentration used in their experiment involving reduction with TMPD, but it was probably substantially lower.

The formate complex of cytochrome oxidase was prepared by adding 1.0 M NaHCOO to the aerobic enzyme solution to a final formate concentration of 100 mM. The resulting solution was incubated for at least 6 h at 4 °C before optical spectra were recorded. The half-reduced formate complex was prepared by adding sodium ascorbate (6 mM) and TMPD (60 μM) to the oxidized formate complex. Optical spectra were recorded after 30 min. Formate does not inhibit the enzyme as effectively as cyanide, so spectra recorded after overnight incubation were significantly different owing to partial reduction of cytochrome a_3. EPR spectra of similarly treated samples showed complete reduction of cytochrome *a* and Cu_A and less than 2% reduction of Cu_B as measured by the intensity of the high-spin iron signal at $g = 6$.

The one-fourth-reduced NO-associated enzyme was prepared by incubating the oxidized enzyme with NaN_3 (100 mM) for 6 h followed by vacuum degassing and addition of NO to a pressure of 0.9 atm, as described by Stevens et al. (1979). Optical spectra were recorded 20 min after addition of NO; incubation at 4 °C for 16 h produced only small changes in the spectrum. The EPR spectrum of samples prepared in this manner displays a characteristic triplet signal associated with Cu_B^{2+} in close proximity to cytochrome a_3^{2+}–NO. The intensity of the cytochrome *a* signal near $g = 3.0$ is unchanged upon formation of the triplet, which indicates that cytochrome *a* is not reduced under these conditions.

The half-reduced NO complex of the enzyme was prepared by reduction of the anaerobic enzyme solution with a small excess of NADH in the presence of phenazine methosulfate (PMS) followed by addition of NO to a pressure of 0.1 atm. After 5 min of incubation at 4 °C, the samples were partially reoxidized by treatment with a 2-fold molar excess of ferricyanide. Optical spectra were recorded immediately. The EPR spectra of similarly treated samples exhibit signals due to cytochrome *a* and Cu_A but show no triplet or high-spin heme

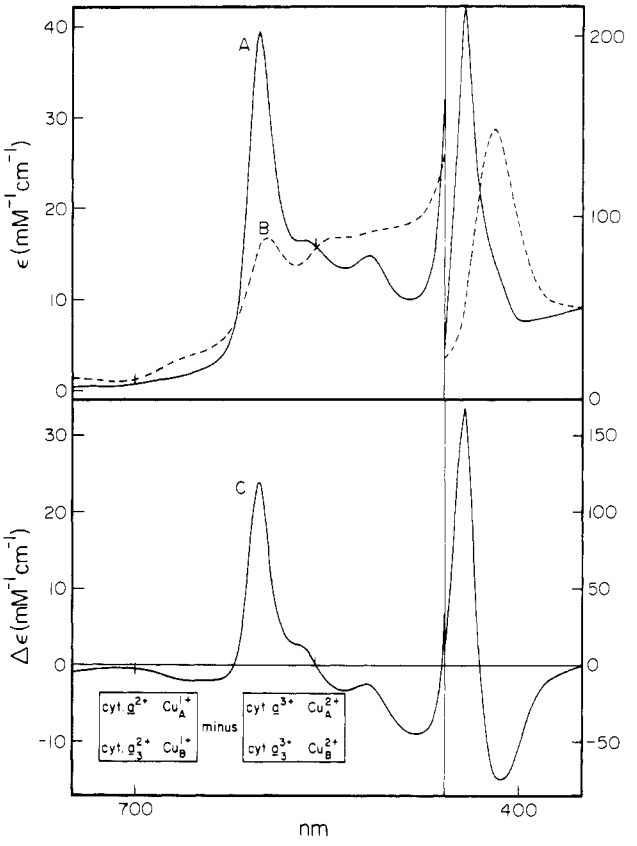

FIGURE 1: Absorption spectra of 15 μM cytochrome oxidase. Trace A, ascorbate reduced; trace B, native oxidized; trace C, reduced minus oxidized (trace A minus trace B).

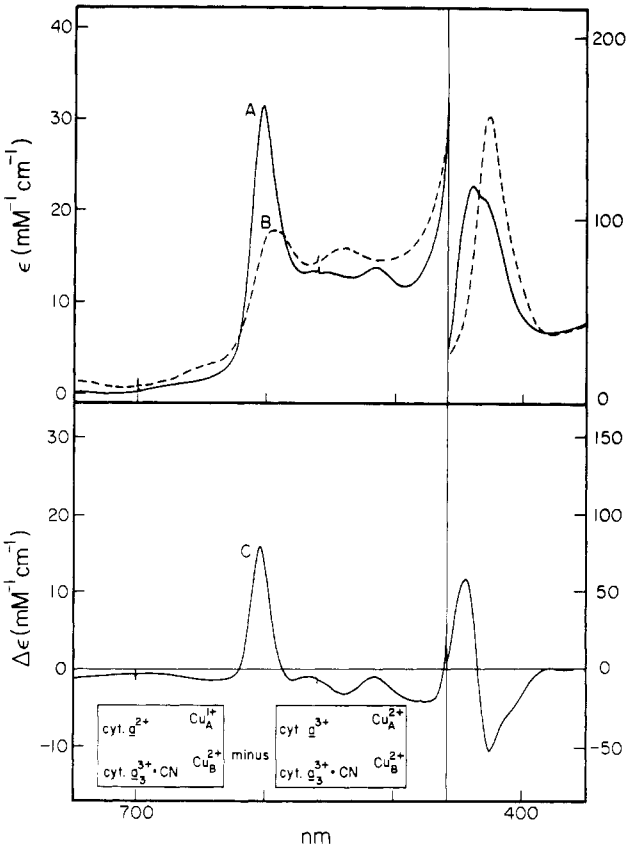

FIGURE 2: Absorption spectra of cyanide-inhibited 15 μM cytochrome oxidase. Trace A, cytochrome oxidase incubated with 100 mM KCN for 4 h at 4 °C followed by treatment with ascorbate and TMPD; trace B, cytochrome oxidase incubated with 100 mM KCN for 4 h at 4 °C; trace C, reduced minus oxidized cytochrome *a*, obtained as the difference of traces A and B.

signals, indicating that Cu_B is not reoxidized by the ferricyanide treatment. Somewhat different results were obtained when high partial pressures (0.9 atm) of NO were employed, probably owing to partial reoxidation of the enzyme by NO via the reaction $2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$ (Brudvig et al., 1980).

The fully reduced NO complex of cytochrome oxidase was prepared by vacuum degassing and reduction of the enzyme with ascorbate (6 mM) and TMPD (60 μM) followed by addition of NO to a pressure of 0.1 atm. Optical spectra were recorded 30 min after addition of NO. EPR spectra recorded under similar conditions showed only the nitrosylferroheme signal associated with the cytochrome a_3^{2+}–NO complex.

Optical spectra were recorded at room temperature with a Beckman Acta CIII spectrophotometer interfaced to a Spex Industries SCAMP SC-31 data processor. Absorption spectra were recorded for each sample and stored on magnetic disk; difference spectra and difference of difference spectra were obtained by computer subtraction. The spectral bandwidth was 1 nm.

EPR spectra were obtained at 12 K with a Varian E-Line Century Series X-band spectrometer equipped with an Air Products Heli-Tran temperature controller. Microwave power was typically 0.2 mW. The enzyme concentration used in the EPR experiments was 165 μM. The intensities of the high-spin heme signals at $g = 6$ were determined by the method of Aasa et al. (1976); that of the cyanocytochrome a_3 EPR signal was integrated by the method of DeVries & Albracht (1979) by using the $g = 3.5$ component to determine the total area.

Results

Absorption spectra of oxidized and reduced cytochrome *c* oxidase are displayed in Figure 1. The composite difference spectrum, which represents reduced minus oxidized cytochrome *a* plus reduced minus oxidized cytochrome a_3, is also displayed in this figure. The composite difference spectrum has peaks at 604 ($\Delta\epsilon$ = 24 mM^{-1} cm^{-1}) and 444 nm ($\Delta\epsilon$ = 168 mM^{-1} cm^{-1}) and isosbestic points at 622, 559, 462, and 431 nm.

When cyanide-inhibited oxidase is treated with ascorbate and TMPD, cytochrome *a* and Cu_A are reduced while Cu_B and cytochrome a_3 remain oxidized. The absorption spectra of the cyanide-inhibited enzyme species are presented in Figure 2. The difference of these spectra, representing reduced minus oxidized cytochrome *a*, is also presented in this figure. The cytochrome *a* difference spectrum obtained by this method has peaks at 605 ($\Delta\epsilon$ = 16 mM^{-1} cm^{-1}) and 444 nm ($\Delta\epsilon$ = 59 mM^{-1} cm^{-1}) and isosbestic points at 619, 585, 461, and 435 nm.

When formate-inhibited cytochrome oxidase is treated with ascorbate and TMPD, cytochrome *a* and Cu_A are reduced while cytochrome a_3 and Cu_B remain oxidized. This mixed valence species is thus similar to that produced when cyanide is used to inhibit the enzyme; however, in the formate complex, cytochrome a_3 remains high spin while in the cyanide complex it is low spin. Absorption spectra of the formate-inhibited mixed valence species and the oxidized formate-inhibited enzyme are displayed in Figure 3; the difference of these spectra, which represents reduced minus oxidized cytochrome *a*, is also shown. This difference spectrum has peaks at 604 ($\Delta\epsilon$ = 17 mM^{-1} cm^{-1}) and 444 nm ($\Delta\epsilon$ = 55 mM^{-1} cm^{-1}) and isosbestic points at 619, 585, 460, and 435 nm. It is thus similar to, but not identical with, the cytochrome *a* difference spectrum obtained by using cyanide inhibition of cytochrome a_3 (Figure 2); the most notable differences occur in the 570- and 415-nm regions.

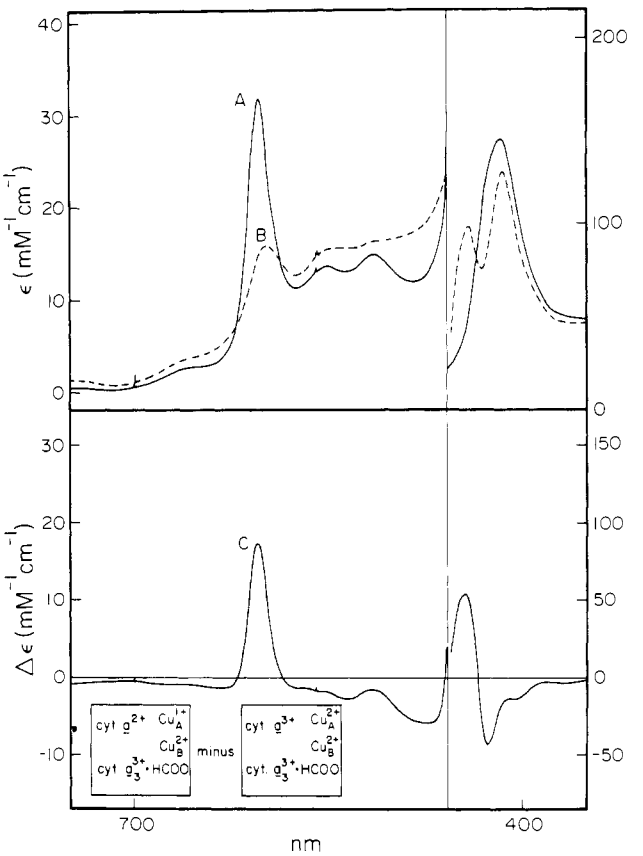

FIGURE 3: Absorption spectra of formate-inhibited 13.6 μM cytochrome oxidase. Trace A, cytochrome oxidase incubated with 100 mM KCHOO for 6 h at 4 °C followed by treatment with ascorbate and TMPD; trace B, cytochrome oxidase incubated with 100 mM KHCOO for 6 h at 4 °C; trace C, reduced minus oxidized cytochrome *a*, obtained as the difference of traces A and B.

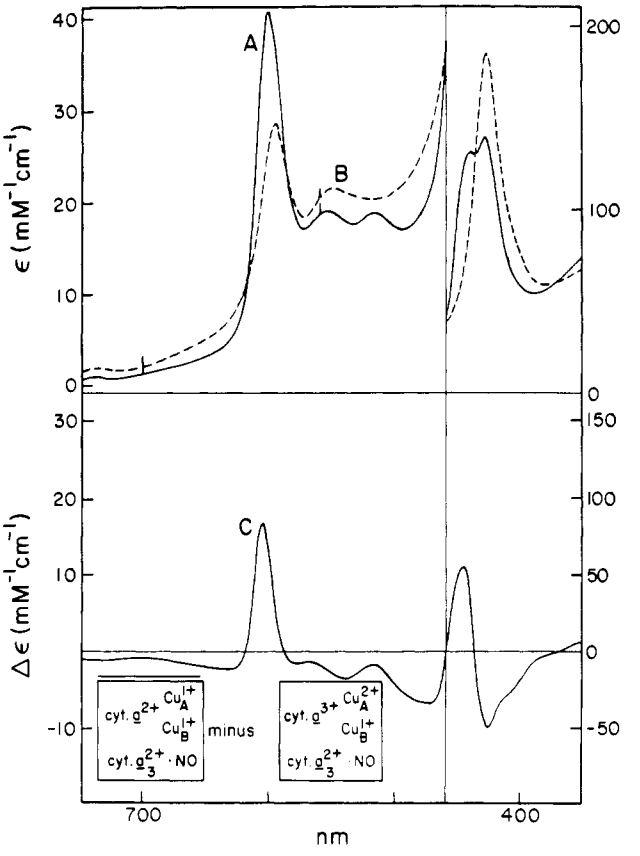

FIGURE 4: Absorption spectra of 12 μM, nitric oxide inhibited cytochrome oxidase. Trace A, cytochrome oxidase reduced with NADH and PMS followed by the addition of NO to a pressure of 0.1 atm; trace B, cytochrome oxidase reduced with NADH and PMS followed by the addition of NO to a pressure of 0.1 atm and treatment with a small excess of ferricyanide; trace C, reduced minus oxidized cytochrome *a*, obtained as the difference of traces A and B.

Nitric oxide (NO) has a very high affinity for reduced cytochrome a_3 (Gibson & Greenwood, 1963; Boelens et al., 1982) and should thus stabilize this cytochrome against oxidation by ferricyanide. A mixed-valence species of cytochrome oxidase was prepared by adding a small excess of ferricyanide to the reduced, NO-associated enzyme. Under these conditions, cytochrome *a* and Cu_A are oxidized while cytochrome a_3 and Cu_B remain reduced. Absorbance spectra of this mixed valence species and of fully reduced NO-associated cytochrome oxidase in the 750–350-nm region are displayed in Figure 4. The difference of these spectra, representing reduced minus oxidized cytochrome *a*, is also presented in Figure 4; the difference spectrum shows peaks at 604 ($\Delta\epsilon$ = 17.4 mM^{-1} cm^{-1}) and 444 nm ($\Delta\epsilon$ = 51 mM^{-1} cm^{-1}) and isosbestic points at 617, 585, 461, and 435 nm.

Incubation of oxidized cytochrome oxidase with azide followed by addition of NO produces an enzyme species in which cytochrome a_3 is selectively reduced and associated with NO while the other metal centers of the enzyme remain oxidized (Stevens et al., 1979). Absorption spectra of this one-fourth-reduced NO-associated enzyme and the fully reduced NO-associated enzyme are displayed in Figure 5 together with their difference. The difference spectrum shows peaks at 604 ($\Delta\epsilon$ = 13.7 mM^{-1} cm^{-1}) and 445 nm ($\Delta\epsilon$ = 53 mM^{-1} cm^{-1}) and isosbestic points at 616, 583, 461, and 436 nm. It is different from all of the cytochrome *a* difference spectra obtained by the other methods, most notably with respect to the very low intensity of its 604-nm peak.

The α- and Soret-band maxima and extinctions of the absolute and difference spectra of the various enzyme species are summarized in Tables I and II. For characterization of

Table I: Band Positions and Extinctions of Cytochrome Oxidase Species

enzyme species	α-band max (nm)	ϵ_α	Soret-band max (nm)	ϵ_{Soret}
oxidized	597	16.3	420	141
reduced	603	39.6	442	209
oxidized + CN^-	594	18.1	427	156
oxidized + CN^- + ascorbate/TMPD	603	31.8	440	118
oxidized + formate	597	15.8	417	145
oxidized + formate + ascorbate/TMPD	603	31.7	415, 442	128, 102
reduced + NO	601	41.3	428, 440	138, 130
reduced + NO + ferricyanide	595	27.7	427	184
oxidized + N_3^- + NO	598	28.9	428	177

the differences between the cytochrome *a* difference spectra obtained by the various methods, the diffference spectra were subtracted from each other pairwise. Some of the difference of difference spectra thus obtained are displayed in Figures 6 and 7. The strongest features of these spectra appear in the 600-nm region and in the 400–450-nm region, which is consistent with assignment to one or both of the cytochromes. Features which deserve comment include the following: A strong, asymmetric peak is found at 606 nm in the half-reduced NO minus one-fourth-reduced NO difference of difference spectrum (Figure 6). There is strong positive intensity centered at 415 nm in the formate minus one-fourth-reduced NO and

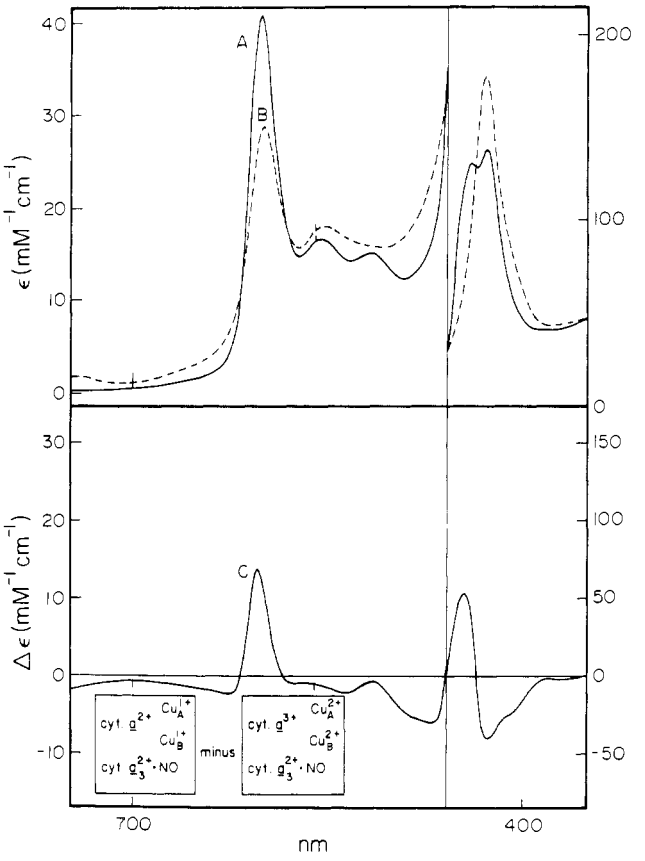

FIGURE 5: Absorption spectra of 11 μM, nitric oxide inhibited cytochrome oxidase. Trace A, cytochrome oxidase reduced with ascorbate and TMPD followed by addition of NO to a pressure of 0.1 atm; trace B, cytochrome oxidase incubated with 100 mM NaN_3 for 6 h at 4 °C followed by addition of NO to a pressure of 0.9 atm and incubation for 20 min at 4 °C; trace C, reduced minus oxidized cytochrome *a*, obtained as the difference of traces A and B.

Table II: Band Positions and Extinctions of Cytochrome Oxidase Difference Spectra

difference spectrum	α-band max (nm)	ϵ_α	Soret-band max (nm)	ϵ_{Soret}
reduced minus oxidized	604	24	444	168
oxidized + CN^- + ascorbate/TMPD minus oxidized + CN^-	605	16	444	59
oxidized + formate + ascorbate/TMPD minus oxidized + formate	604	17	444	55
reduced + NO minus reduced + NO + ferricyanide	604	17.4	444	56
reduced + NO minus oxidized + N_3^- + NO	604	13.7	445	53

formate minus cyanide difference of difference spectra (Figure 7). Intense derivative-shaped features suggestive of ca. 3-nm peak shifts are found near 430 nm in many of the Soret region difference of difference spectra. Finally, it should be noted that the features in both the 600-nm region and the Soret region are sometimes complex and in most cases do not resemble the reduced minus oxidized spectra of cytochrome *a*.

Discussion

In the simplest model of the cytochrome oxidase absorption spectrum, the metal centers are assumed to be spectrally independent; i.e., the absorbance of each of the metal centers

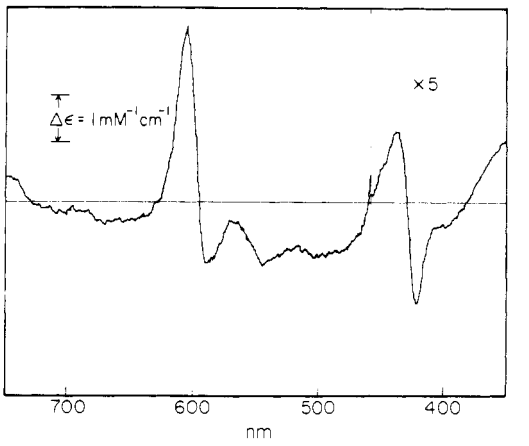

FIGURE 6: Difference of difference spectrum formed from the cytochrome *a* difference spectra obtained by the one-fourth-reduced NO method and the half-reduced NO method. This spectrum is equivalent to the one-fourth-reduced NO complex minus the half-reduced NO complex difference spectrum since these two methods employ the same reference state, i.e., the fully reduced NO complex, in calculating the cytochrome *a* difference spectrum.

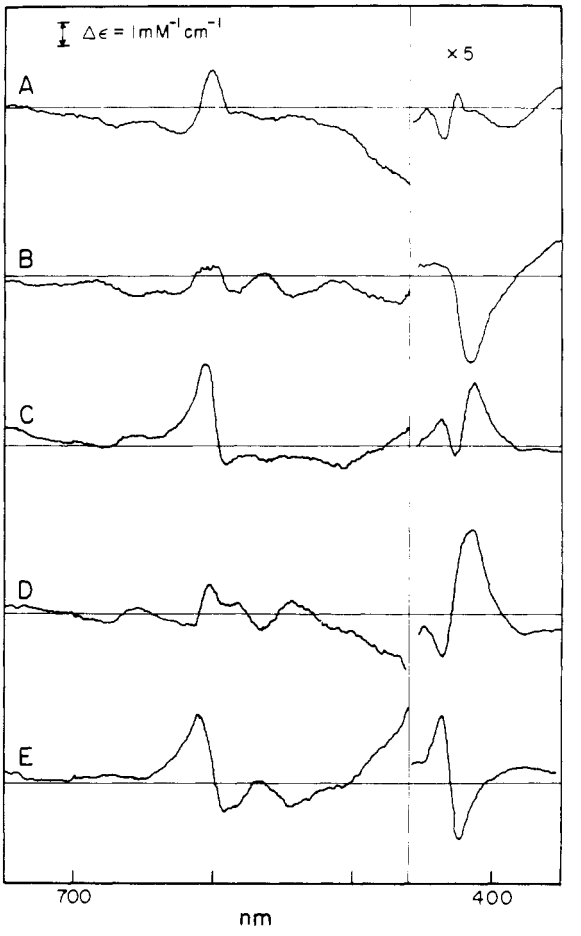

FIGURE 7: Difference of difference spectra formed from the cytochrome *a* difference spectra obtained by various methods. Trace A, mixed-valence NO method minus cyanide method (trace 4C minus trace 1C); trace B, mixed-valence NO method minus formate method (trace 4C minus trace 2C); trace C, formate method minus triplet method (trace 2C minus trace 5C); trace D, formate method minus cyanide method (trace 2C minus trace 1C); trace E, cyanide method minus triplet method (trace 1C minus trace 5C).

is considered to be independent of the oxidation or ligation state of the other metal centers in the enzyme. Within this framework, the composite cytochrome absorption may be expressed as a sum:

$$\epsilon(\lambda) = \epsilon_a^{o}(\lambda) + \epsilon_{a_3}^{o}(\lambda) + f_a\Delta\epsilon_a^{r-o}(\lambda) + f_{a_3}\Delta\epsilon_{a_3}^{r-o}(\lambda)$$

where $\epsilon_a^{o}(\lambda)$ and $\epsilon_{a_3}'(\lambda)$ are the extinctions of oxidized cytochromes a and a_3, respectively, f_a and f_{a_3} denote the fractions of cytochromes a and a_3 which are reduced, and $\Delta\epsilon_a^{r-o}(\lambda)$ and $\Delta\epsilon_{a_3}^{r-o}(\lambda)$ refer to the changes in extinction which accompany reduction of the two cytochromes. It has been assumed that the copper centers make a negligible contribution to the enzyme absorption in the spectral range of interest (350–700 nm) (Bocian et al., 1979). Cytochrome a_3 may be stabilized in either the reduced ($f_{a_3} = 1.0$) or the oxidized ($f_{a_3} = 0$) state by exogenous ligands while cytochrome a is reduced. The absorbance change accompanying reduction of cytochrome a [$\Delta\epsilon_a^{r-o}(\lambda)$] may thus, under these assumptions, be measured without interfering contributions from cytochrome a_3.

If the metal centers are not spectrally independent, new terms must be incorporated in this equation to describe the effects of redox and/or ligation state of each of the metal centers upon the absorption properties of each of the cytochromes. The equation then becomes

$$\epsilon(\lambda) = \epsilon_a^{o}(\lambda) + \epsilon_{a_3}^{o}(\lambda) + f_a\Delta\epsilon_a^{r-o}(\lambda) + f_{a_3}\Delta\epsilon_{a_3}^{r-o}(\lambda) + \Delta\epsilon_a^{i}(\lambda,a_3,\mathrm{A},\mathrm{B}) + \Delta\epsilon_{a_3}^{i}(\lambda,a,\mathrm{A},\mathrm{B})$$

where $\Delta\epsilon_a^{i}(\lambda,a_3,\mathrm{A},\mathrm{B})$ and $\Delta\epsilon_{a_3}^{i}(\lambda,a,\mathrm{A},\mathrm{B})$ describe any metal–metal interactions which influence the absorption properties of reduced or oxidized cytochromes a and a_3, respectively.

We have applied four different methods, all based upon inhibition of cytochrome a_3 by exogenous ligands, to the estimation of the reduced minus oxidized difference spectrum of cytochrome a. Each method gives a different result, especially with respect to the intensity of the 604-nm peak, for which the estimated value of $\Delta\epsilon_a^{r-o}$ varied between 13.7 and 17.4 mM^{-1} cm^{-1}. At the same time, the intensity of the 444-nm peak exhibited much less variability. Our estimates for the intensity of this peak ranged from 53 to 59 mM^{-1} cm^{-1}. The Soret region nevertheless displayed a substantial degree of variability in the 415-nm region [compare, for example, the cyanide method (Figure 2) and the formate method (Figure 3)] and in the 430-nm region [cf. cyanide minus one-fourth-reduced NO difference of difference spectrum (Figure 6)].

It might be argued that the variability in cytochrome a difference spectra which we observe is a simple consequence of sample heterogeneity, for example, incomplete reduction of cytochrome a in the mixed-valence cyanide complex or partial reduction of cytochrome a in the triplet species. However, most such possibilities are ruled out by the characterization of our samples by EPR spectroscopy (see Materials and Methods). Partial reduction of cytochrome a in the oxidized cyanide- or formate-inhibited oxidase ($f_a \neq 0$) would result in a corresponding underestimate of the cytochrome a reduced minus oxidized difference spectrum. However, ferricyanide addition, which should reoxidize cytochrome a, to samples of cyanide-inhibited oxidase prepared as in Figure 2 does not cause any changes in the optical spectrum except the appearance of the ferricyanide absorption near 420 nm (data not shown). Formate is a much less effective inhibitor of the oxidase than is cyanide, so under the aerobic conditions employed, autoreduction of cytochrome a is even less likely than in the case of the cyanide experiment. Incomplete binding of formate to the oxidized enzyme, which might be relieved upon the addition of reductant and cycling of the enzyme, would add an extra component to the cytochrome a reduced minus oxidized spectrum obtained by the formate method. This additional component would be expected to resemble the formate-complexed minus native-oxidized difference spectrum. This difference spectrum (not shown) exhibits a peak at 411 nm and a trough at 424 nm and is approximately 5-fold less intense than the strong positive features near 416 nm in the difference of difference spectra involving the formate method. The binding of formate to the resting oxidized enzyme must therefore be only about 20% complete if incomplete binding is responsible for these features. Preliminary studies in our laboratory suggest that formate may in fact bind more completely to the "pulsed" enzyme than to the resting enzyme (data not shown); more experiments are under way to determine whether this effect will fully explain the features near 416 nm in our difference of difference spectra. It should be emphasized that many of the features observed in these spectra do not resemble the formate-complexed minus native-oxidized difference spectrum.

The differences which we observe between the cytochrome a difference spectra obtained by the various methods are not those expected if sample heterogeneity is the cause. Thus, while the one-fourth-reduced NO method and the half-reduced NO method give substantially different reduced minus oxidized intensities at 604 nm, they are in much closer agreement with respect to the intensity at 444 nm. In most cases, the difference of difference spectra in the 600-nm region do not resemble the cytochrome a reduced minus oxidized difference spectra; typically, their shapes are suggestive of shifts in peak position as well as changes in peak intensities. In the Soret region, the difference of difference spectra do not resemble the reduced minus oxidized spectra of cytochrome a or that expected for cytochrome a_3 but are instead suggestive of shifts in position or changes in intensity of peaks associated with species of defined redox state.

The observed spectral variations are thus most plausibly explained as arising from interaction between the metal centers of the enzyme ($\Delta\epsilon_a^{i} \neq 0$ or $\Delta\epsilon_{a_3}^{i} \neq 0$). We will now discuss the evidence which bears upon the nature and mechanism of this interaction.

Any metal–metal interaction which influences the extinction of cytochrome a is expressed in the term $\Delta\epsilon_a^{i}(\lambda,a_3,\mathrm{A},\mathrm{B})$, and any which influences the extinction of cytochrome a_3 is expressed in the term $\Delta\epsilon_{a_3}^{i}(\lambda,a,\mathrm{A},\mathrm{B})$. Conceivably, either or both of these terms may contribute to the observed differences, and any of the variables in parentheses may be involved. Fortunately, the difference of difference spectra in Figure 6 furnish some clues as to which term(s) and which variable(s) are important.

In the triplet species, Cu_B is oxidized while cytochrome a_3 is reduced and associated with NO. In the half-reduced NO-associated species, both cytochrome a_3 and Cu_B are reduced. The oxidation state of Cu_B is thus the only feature which distinguishes these species; nevertheless, the two methods involving these complexes give the most discrepant results with respect to the intensity of the 604-nm peak in the cytochrome a difference spectrum. The oxidation state of Cu_B must therefore be important in determining the absorption properties of the enzyme. The difference of difference spectrum comparing these two methods (Figure 6), which is equivalent to a one-fourth-reduced NO minus half-reduced NO difference spectrum, shows a fairly strong, asymmetric peak at 606 nm. The intensity and width of this peak indicate that it is not associated with a transition of a copper ion (blue copper charge-transfer transitions are typically 3–4 times more broad; Solomon et al., 1980) but point instead to the involvement of one or both of the cytochromes.

The α-band extinction of both high-spin and low-spin heme a model compounds is known to be sensitive to solvent polarity

(Babcock et al., 1979, 1981). Since Cu_B is fairly close to cytochrome a_3 (Tweedle et al., 1978; Stevens et al., 1979), the redox state of Cu_B might be expected to influence the extinction of cytochrome a_3. Davis et al. (1981) have found that a charge situated on the periphery of the chlorophyll *a* macrocycle can induce a reversible wavelength shift of 90 cm^{-1} and an increase in extinction of approximately 8 mM^{-1} cm^{-1} in the red absorption maximum of this chromophore. In the case of the cytochrome a_3–NO complex, a red shift of comparable magnitude (ca. 70 cm^{-1}) is observed upon oxidation of Cu_B (compare Figures 4 and 5), suggesting that cytochrome a_3 is influenced by the additional charge on Cu_B. The oscillator strength of the α band is expected to increase if the perturbation due to this charge has the appropriate symmetry for mixing the α-band excited state with the excited state of the more strongly allowed Soret transition. The difference of difference spectrum comparing the one-fourth-reduced NO complex to the half-reduced NO complex is plausibly explained as arising from the term $\Delta\epsilon_{a_3}{}^{i}(\lambda,a,A,B)$, in which the critical variable is B; the low apparent value for $\Delta\epsilon_a$ obtained by using the one-fourth-reduced NO method is in fact due to the influence of Cu_B upon cytochrome a_3 rather than to the properties of cytochrome *a* itself. While we cannot rule out the possibility that the term $\Delta\epsilon_a{}^{i}(\lambda,a_3,A,B)$ also contributes to the spectra of the NO complexes, this term does not appear necessary.

An interaction between Cu_B and cytochrome a_3 is made plausible by the proximity of these sites within the enzyme. However, this kind of interaction alone will not account for all of the data. The discrepancies between the cyanide, formate, and half-reduced NO methods suggest that there may be an interaction between the "a sites" (cytochrome *a* and Cu_A) and the metals of the oxygen-binding site (cytochrome a_3 and Cu_B). In the absence of such an interaction, we would expect agreement between these methods. Again, the observed discrepancies may involve either $\Delta\epsilon_a{}^{i}(a_3,A,B,\lambda)$ or $\Delta\epsilon_{a_3}{}^{i}(a,A,B,\lambda)$; for a number of reasons, we favor interpretation in terms of $\Delta\epsilon_{a_3}{}^{i}(a,A,B,\lambda)$.

First, the Soret region difference of difference spectra which involve the formate method (Figure 7, traces B, C and D) show a strong feature near 415 nm. The other Soret region difference of difference spectra do not have this feature. The ca. 415-nm peak is therefore associated with the formate method; the cytochrome *a* reduced minus oxidized difference spectrum obtained by this method (Figure 3) in fact exhibits substantially lower negative intensity in this region than do the other methods. The negative intensity of this spectrum at 415 nm is only about 15% of the composite (cytochrome *a* plus cytochrome a_3) reduced minus oxidized intensity at this wavelength. Given that the 415-nm feature in the Soret region difference of difference spectra is associated with the formate method, the question remains as to which cytochrome extinction is involved. Since cytochrome a^{3+} absorbs most strongly at longer wavelengths (ca. 427 nm), it is probably not responsible for the 415-nm feature; the same is true for cytochrome a^{2+}, which absorbs near 442 nm. The cytochrome a_3–formate complex, on the other hand, has its absorption maximum at 416 nm and may thus be associated with the 415-nm feature. As noted above, incomplete (ca. 20%) binding of formate to the resting enzyme, which is relieved by the addition of reductant, might be responsible for this feature. Another possibility is that the reduction of cytochrome *a* and/or Cu_A causes an increase in the extinction at 415 nm of the cytochrome a_3–formate complex, leading to an underestimate of the cytochrome *a* reduced minus oxidized intensity at this wavelength. Presumably, the α band is influenced by a similar mechanism, leading to some of the discrepancies observed in this region.

The difference of difference spectra in the Soret region also exhibit features suggestive of small (ca. 4 nm) peak shifts in the neighborhood of 430 nm, which appear to be associated with both the one-fourth-reduced NO method and the cyanide method. As note above, cytochrome a^{3+} absorbs maximally near 427 nm, which might suggest the involvement of this cytochrome. However, the cytochrome $a_3{}^{3+}$–CN complex and the cytochrome $a_3{}^{2+}$–NO complex also absorb near 427 nm. The features at 430 nm may therefore also be associated with ligand complexes of cytochrome a_3 rather than cytochrome *a* and hence with the term $\Delta\epsilon_{a_3}{}^{i}(\lambda,a,A,B)$ rather than the term $\Delta\epsilon_a{}^{2}(\lambda,a_3,A,B)$.

Finally, an interaction between the a sites and the oxygen-binding site which influences the extinction of cytochrome a_3 rather than that of cytochrome *a* is made attractive by the demonstrated sensitivity of cytochrome a_3's absorption spectrum to its environment (specifically, the redox state of Cu_B). This sensitivity suggests a plausible mechanism whereby the extinction of cytochrome a_3 may be modulated by the states of the other metal centers: reduction of cytochrome *a* and/or Cu_A may induce a conformational change which causes Cu_B to move in relation to cytochrome a_3 and so influence its extinction. The redox and ligation state of cytochrome a_3 will determine the nature and extent of this effect, causing the observed discrepancies between the cyanide, formate, and NO methods.

We may thus account for all of the data in a model involving one interaction term $[\Delta\epsilon_{a_3}{}^{i}(a,A,B,\lambda)]$, and treating cytochrome *a* as spectrally isolated. The reduced minus oxidized difference spectrum of cytochrome *a* nevertheless remains undetermined in some important respects. The α-band intensity of this difference spectrum is probably between 16 and 17.4 mM^{-1} cm^{-1} (the range established by the three techniques in which the redox state of Cu_B did *not* vary and which should therefore minimize interaction effects). Cytochrome *a* thus contributes between 67% and 73% to the total reduced minus oxidized absorbance near 600 nm. The Soret peak at 444 nm is more closely determined, lying somewhere between 55 and 59 mM^{-1} cm^{-1}. This value is substantially lower than many previous estimates but agrees well with recent results (F. G. Halaka, J. L. Dye, and G. T. Babcock, unpublished experiments). It appears probable that in earlier experiments with cyanide, inhibition of cytochrome a_3 (and/or Cu_B) was incomplete. We have found that substantially higher estimates of $\Delta\epsilon_a$ are obtained if low cyanide concentrations (2 mM) and short incubation times ($\sim$1 min) are employed (data not shown).

Some useful features are conserved in the cytochrome *a* difference spectra obtained with all of the methods, including isosbestic points near 435 and near 585 nm (the latter moves to 583 nm in the triplet case, which, as noted above, is expected to be the least accurate representation of the cytochrome *a* difference spectrum). Because of the presence of these isosbestic points, the cytochrome oxidase absorption spectrum retains some utility for the monitoring of electrochemical titrations; in fact, the pronounced influence of Cu_B's redox state upon the extinction of cytochrome a_3 may make it possible to monitor this otherwise invisible site along with the cytochromes.

We have suggested that the reduction of cytochrome *a* and/or Cu_B might induce a conformational change which changes the position of Cu_B in relation to cytochrome a_3, thereby influencing the extinction of cytochrome a_3, probably

via an electrostatic mechanism. The cytochrome–cytochrome redox potential interaction which has been proposed (Wikström et al., 1976; Carithers & Palmer, 1981) may involve the same mechanism since the position of Cu_B and its associated charge(s) in relation to cytochrome a_3 might influence the redox potential of the latter. In this connection, it should be noted that a redox potential interaction between Cu_B and cytochrome a_3, which has been less frequently proposed than a cytochrome–cytochrome interaction, is also expected in this case if the unperturbed reduction potentials of cytochrome a_3 and Cu_B are sufficiently close.

References

Aasa, R., Albracht, S. P., Lanne, B., & Vänngard, T. (1976) *Biochim. Biophys. Acta 422*, 260–272.

Babcock, G. T., Vickery, L. E., & Palmer, G. (1976) *J. Biol. Chem. 251*, 7907–7919.

Babcock, G. T., Vickery, L. E., & Palmer, G. (1978) *J. Biol. Chem. 253*, 2400–2411.

Babcock, G. T., Ondrias, M. R., Gobeli, D. A., van Steelandt, J., & Leroi, G. E. (1979) *FEBS Lett. 108*, 147.

Babcock, G. T., Callahan, P. M., Ondrias, M. R., & Salmeen, I. (1981) *Biochemistry 20*, 959–966.

Blokzijl-Homan, M. F. J., & Van Gelder, B. F. (1971) *Biochim. Biophys. Acta 234*, 493–498.

Blumberg, W. E., & Peisach, J. (1979) *Dev. Biochem. 5*, 153–159.

Bocian, D. F., Lemley, A. T., Peterson, N. O., Brudvig, G. W., & Chan, S. I. (1979) *Biochemistry 18*, 4396–4402.

Boelens, R., Rademaker, H., Pel, R., & Wever, R. (1982) *Biochim. Biophys. Acta 679*, 84–94.

Brudvig, G. S., Stevens, T. H., & Chan, S. I. (1980) *Biochemistry 19*, 5275.

Carithers, R. P., & Palmer, G. (1981) *J. Biol. Chem. 256*, 7967–7976.

Davis, R. C., Ditson, S. L., Fentiman, A. F., & Pearlstein, R. M. (1981) *J. Am. Chem. Soc. 103*, 6823–6826.

DeVries, S., & Albracht, S. P. J. (1979) *Biochim. Biophys. Acta 546*, 334–340.

Gibson, Q. H., & Greenwood, C. (1963) *Biochem. J. 86*, 541–554.

Hartzell, C. R., & Beinert, H. (1974) *Biochim. Biophys. Acta 368*, 318–338.

Horie, S., & Morrison, M. (1963) *J. Biol. Chem. 238*, 2859–2868.

Johnson, M. K., Eglinton, D. G., Goodig, P. E., Greenwood, C., & Thomson, A. J. (1981) *Biochem. J. 193*, 699–708.

Keilin, D., & Hartree, E. F. (1939) *Proc. R. Soc. London, Ser. B 127*, 167–191.

Leigh, J. S., Wilson, D. F., Owen, C. S., & King, T. E. (1974) *Arch. Biochem. Biophys. 160*, 476–487.

Mackay, L. N., Kuwana, T., & Hartzell, C. R. (1973) *FEBS Lett. 36*, 326–329.

Malmström, B. (1979) *Biochim. Biophys. Acta 549*, 281–303.

Nicholls, P. (1976) *Biochim. Biophys. Acta 430*, 13–29.

Schroedl, N. A., & Hartzell, C. R. (1977a) *Biochemistry 16*, 4966–4971.

Schroedl, N. A., & Hartzell, C. R. (1977b) *Biochemistry 16*, 4961–4965.

Solomon, E. I., Hare, J. W., Dooley, D. M., Dawson, J. H., Stephens, P. J., & Gray, H. B. (1980) *J. Am. Chem. Soc. 102*, 169–178.

Stevens, T. H., Brudvig, G. W., Bocian, D. F., & Chan, S. I. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3320–3324.

Tiesjema, R. H., Muijsers, A. O., & Van Gelder, B. F. (1973) *Biochim. Biophys. Acta 305*, 19–28.

Tweedle, M. F., Wilson, L. J., García-Iñiguez, L., Babcock, G. T., & Palmer, G. (1978) *J. Biol. Chem. 253*, 8065–8071.

Van Gelder, B. F. (1966) *Biochim. Biophys. Acta 118*, 36.

Vanneste, W. H., & Vanneste, M.-T. (1965) *Biochem. Biophys. Res. Commun. 19*, 182–186.

Wikström, M. K. F., Harmon, H. J., Ingledew, W. J., & Chance, B. (1976) *FEBS Lett. 65*, 259–277.

Wilson, D. F., & Leigh, J. S. (1974) *Ann. N.Y. Acad. Sci. 227*, 630–635.

Wilson, D. F., Lindsay, J. G., & Brocklehurst, E. S. (1972) *Biochim. Biophys. Acta 256*, 277–286.

Yonetani, T. (1960) *J. Biol. Chem. 235*, 845–852.