Communication

Chemical and Spectroscopic Evidence for the Formation of a Ferryl Fe_{a_3} Intermediate during Turnover of Cytochrome c Oxidase*
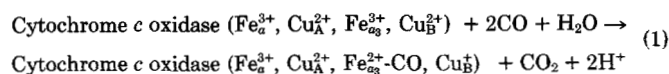
Stephan N. Witt, David F. Blair‡, and Sunney I. Chan§

From the A. A. Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91125

When partially reduced cytochrome c oxidase samples are reoxidized with dioxygen, an EPR-silent dioxygen intermediate, which is at the three-electron level of dioxygen reduction, is trapped at the dioxygen reduction site. The intermediate has novel spectral features at 580 and 537 nm. Combined optical and EPR results reveal that this intermediate reacts rapidly with CO at 277–298 K causing the abolition of the 580/537 nm features and the appearance of a rhombic Cu_B EPR signal. A ferryl Fe_{a_3}, or an intermediate at the same formal level of oxidation, is proposed to oxidize CO to CO_2 producing an EPR-detectable Cu_B adjacent to a low-spin ferrous Fe_{a_3}-dioxygen (or carbon monoxide) adduct.

It is now generally recognized that carbon monoxide can reduce cytochrome c oxidase to produce the CO-mixed-valence compound by the following reaction (Equation 1) (1–4).

$$\text{Cytochrome } c \text{ oxidase } (Fe_a^{3+}, Cu_A^{2+}, Fe_{a_3}^{3+}, Cu_B^{2+}) + 2CO + H_2O \rightarrow$$
$$\text{Cytochrome } c \text{ oxidase } (Fe_a^{3+}, Cu_A^{2+}, Fe_{a_3}^{2+}\text{-}CO, Cu_B^{+}) + CO_2 + 2H^+ \quad (1)$$

The rate of the reaction depends on whether the enzyme is in the resting or pulsed state. The reaction between the resting enzyme and CO is relatively slow, with a half-time ($t_{1/2}$) of 400 min at 277 K (4). In contrast, the reaction with the pulsed form of the oxidized enzyme occurs much more rapidly under identical conditions ($t_{1/2}$ = 4 min at 277 K) (4). It has been suggested that the different reactivities exhibited by the resting and pulsed oxidase toward various ligands reflect the different states of ligation at the dioxygen reduction site in these two forms of the oxidized enzyme (5, 6).

In this communication we report evidence for a second reaction between cytochrome c oxidase and CO. Recently, we reported evidence for *two* cytochrome c oxidase intermediates at low temperatures, each involving dioxygen at the three-

electron level of reduction (7). The two different intermediates are formed in sequence: the first intermediate, which is EPR-detectable, subsequently decays to a second intermediate that is EPR-silent under the conditions of our experiments. It was proposed that the first, EPR-detectable intermediate is a hydroperoxide bridged cupric Cu_B/ferrous Fe_{a_3} adduct at the dioxygen reduction site, and the second intermediate a ferryl Fe_{a_3}/cupric Cu_B couple, formed by cleavage of the O—O bond in the hydroperoxide adduct. We also reported that the second intermediate reacted at relatively low temperature (211 K) with an electron donor to produce a species at the binuclear site with a rhombic Cu_B EPR signal. This rhombic Cu_B EPR signal is characteristic of a species in which Fe_{a_3} is stabilized in the ferrous low-spin state by the coordination of dioxygen (or possibly carbon monoxide). Since CO was present in high concentrations as an inhibitor in these experiments, it was reasonably concluded that CO was the electron donor.

The purpose of this communication is to provide further evidence for the proposed ferryl Fe_{a_3}/cupric Cu_B intermediate and to characterize its reaction with CO more fully. In the present work, we have trapped the EPR-silent, three-electron reduced, dioxygen intermediate by reoxidizing partially reduced samples with dioxygen at 277–298 K, instead of fully reduced samples at much lower temperatures as in our previous experiments (7). We have recorded the optical spectrum of this intermediate and have studied its reaction with CO by EPR and optical spectroscopy.

EXPERIMENTAL PROCEDURES

Beef heart cytochrome c oxidase was prepared by the method of Hartzell and Beinert (8). The enzyme was dissolved in 50 mM phosphate buffer containing 0.5% (w/v) Tween-20 at pH 7.4. Enzyme concentrations for the EPR experiments were typically 150–250 μM. EPR samples for the trapping experiments were prepared in 3.8 mm, outer diameter, quartz EPR tubes fitted with vacuum line joints. Samples were deoxygenated by repeated flushing with argon which had been passed through a 1-m long scrubbing column containing manganese dioxide supported on vermiculite. Appropriate amounts of deoxygenated NADH (Sigma) were added to achieve the desired extent of reduction. Phenazine methosulfate (PMS) (0.01 eq) and horse heart cytochrome c (0.001–0.003 eq, Sigma type VI) were added to facilitate reduction via one-electron mediation. After partial or complete reduction, samples were incubated at ice temperature for a minimum of 2 h to allow for complete redox equilibration. Samples were then frozen to 77 K and EPR spectra were recorded at low temperatures (~10 K).

To initiate the reoxidation of the partially reduced samples, samples were evacuated while frozen at 77 K and 1 atm of dioxygen was admitted. The samples were then thawed by immersion in a water bath at room temperature. The thawed samples were shaken and immediately refrozen to 77 K. The interval from thawing to refreezing was made as short as possible to minimize the loss of any relatively unstable trapped intermediates. Typical intervals between thawing and refreezing were 30–45 s.

To initiate the reaction of the reoxidized samples with CO, carbon monoxide (99.99% pure, Matheson Co.), or argon as a control, was admitted to the samples according to the procedure described above. Low temperature EPR was used to ascertain whether a reaction had occurred between the reoxidized samples and CO. When CO was added to reoxidized samples that were initially reduced from 60 to 90%, a rhombic Cu_B EPR signal ($A_\parallel = 10.5$ mT, $g_\parallel = 2.29$) was observed. Such a signal has been reported previously (9, 10), although under different reaction conditions, and was attributed to a cupric Cu_B adjacent to a ferrous Fe_a-dioxygen adduct (11). The intensity of the rhombic Cu_B EPR signal was determined by the method of Aasa and Vänngård using the $g = 3$ component of ferric Fe_a as an internal
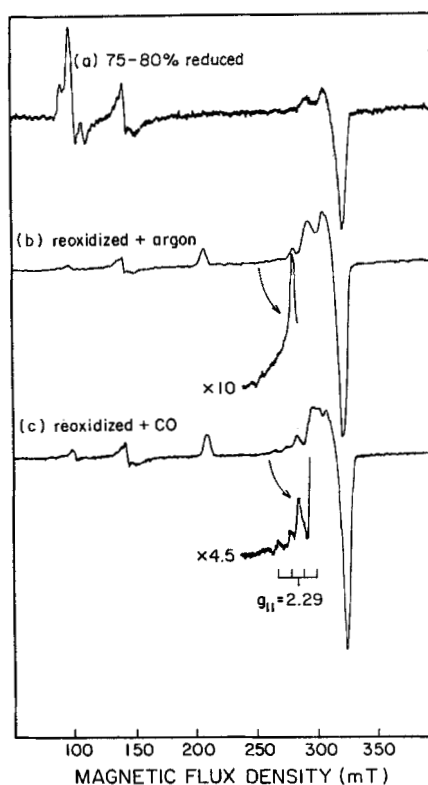
FIG. 1. **EPR spectra of partially reduced and reoxidized cytochrome c oxidase.** A sample that was initially reduced by 75–80% (3.0–3.2 eq) (*a*) and reoxidized with 1 atm of O_2 was incubated with 1 atm of argon, as a control, for 30–45 s and then frozen to 77 K (*b*) and then with 1 atm of CO (*c*) for 30–45 s at 298 K before refreezing. The *insets* beneath *b* and *c*, respectively, show the $g = 2.3$ region at increased power and gain. The *inset* to *b* verifies that no rhombic Cu_B EPR signal was produced in the control. Conditions for obtaining EPR spectra were: temperature, 16 K; microwave power, 0.02 mW (*a–c*), 0.2 mW (*b, inset*), and 0.04 mW (*c, inset*); and gain, 3.2×10^4 (*a*), 8×10^3 (*b*), 2.5×10^4 (*b, inset*), 1×10^4 (*c*), and 3.2×10^4 (*c, inset*).

standard (12). The g values used in the integration of the Cu_B EPR signal were determined from computer simulations of the spectrum (9).

EPR spectra were recorded on a Varian E-line Century series X-band spectrometer. Either an Oxford low temperature system or a Air Products Heli-Trans low temperature system was used for temperature control. A modulation amplitude of 1.6 millitesla and a modulation frequency of 100 kHz were used to obtain the spectra. The microwave power was typically 0.02 mW.[1] A carbon-glass resistor was used to measure the temperature inside the cavity after each spectrum was recorded. To facilitate data manipulation, the analog data were digitized and collected on a Compaq-Plus PC.

For the optical experiments, samples were degassed and reduced in precision 2-mm path length cells using a modification of the methods described above. Typical enzyme concentrations were 70–80 μM. After the samples were partially reduced, they were incubated for 4–12 h at ice temperature to ensure redox equilibrium. The fully reduced sample was prepared by reducing with excess NADH containing approximately 0.02 eq of phenazine methosulfate and then was incubated for 96 h at ice temperature to ensure complete reduction. The samples were then evacuated and 1 atm of dioxygen was admitted as before.

Following reoxidation of the partially or fully reduced samples with dioxygen, carbon monoxide was admitted to reoxidized samples at 277 K within 10–15 min. No freeze-thaw cycles were necessary for the UV-VIS experiments. Optical spectra of the reoxidized samples before and after addition of CO were recorded at 275 K on a Beckman Acta CIII which was interfaced to a Spex Industries SC-31 SCAMP data processor.

[1] The abbreviation used is: mW, milliwatt.

RESULTS

EPR—A typical EPR spectrum for a cytochrome c oxidase sample that was approximately 75–80% reduced is shown in Fig. 1*a*. The intensity of the $g = 6$ component of the high-spin Fe_{a_3} EPR signal provides an indication of the level of reduction. After the partially reduced sample was reoxidized at 277 K, it exhibited the typical Fe_a and Cu_A EPR signals with the expected intensities when the sample was refrozen (spectrum not shown). The very anisotropic Cu_B EPR signal attributed to the hydroperoxide-bridged cupric Cu_B/ferrous Fe_{a_3} adduct (7) was *not* observed at 10 K even with 20 mW of microwave power, nor was the readily saturated rhombic Cu_B EPR signal (7, 9, 10). If, as a control, argon was admitted to the reoxidized sample, there was no evidence of the rhombic Cu_B EPR signal (Fig. 1*b*). When CO was admitted to the same reoxidized sample, however, the rhombic Cu_B EPR signal appeared (Fig. 1*c*). These observations confirm the conclusions of Blair *et al.* (7) that the species exhibiting the rhombic Cu_B EPR signal was produced via a reaction of an EPR-silent intermediate with CO.

In a series of samples we attempted to correlate the yield of the rhombic Cu_B EPR signal with the initial level of reduction. When a half-reduced sample (Fig. 2*a*) was reoxidized and CO admitted, no rhombic Cu_B EPR signal was observed (Fig. 2*b*). Negative results were also obtained when the sample was initially 100% reduced (spectrum not shown). However, when an 80% reduced sample (Fig. 2*c*) was reoxidized and incubated with CO we observed the rhombic Cu_B EPR signal (Fig. 2*d*). The intensity corresponds to 7% of the total Cu_B. These EPR results demonstrate that the species with the rhombic Cu_B EPR signal is formed when CO is added
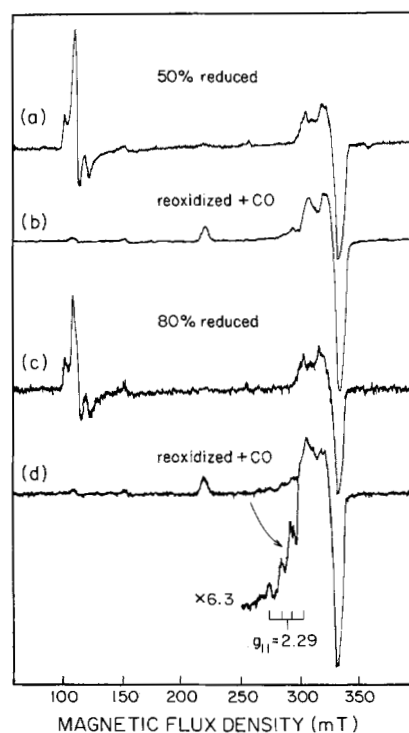

FIG. 2. **EPR spectra of partially reduced and reoxidized cytochrome c oxidase.** Samples that were initially reduced by 50% (2 eq) (*a*) and 80% (3.2 eq) (*c*) and reoxidized with 1 atm of O_2 incubated with 1 atm of CO at 298 K (*b* and *d*) for 30–45 s before freezing. The *inset* below Fig. 2*d* shows the $g = 2.3$ region of the spectrum at a 10-fold higher power and a 2-fold higher gain. Conditions for obtaining EPR spectra were: temperature, 10–13 K; microwave power, 0.02 mW (*a–d*) and 0.20 mW (*d, inset*); gain, 1.6×10^4 (*a*), 1.0×10^4 (*b*), 4.0×10^4 (*c*), 2.0×10^4 (*d*), and 4×10^4 (*d, inset*).

to a reoxidized sample that had been initially reduced by >2 but <4 eq.

Optical—Although EPR spectroscopy has allowed us to monitor a paramagnetic product formed by a reaction between CO and the three-electron reduced dioxygen intermediate, the method does not shed light on the nature of the intermediate, apart from its reactivity with CO, since this species is apparently EPR-silent. We have therefore carried out parallel optical experiments on the reoxidized cytochrome *c* oxidase samples to augment the EPR studies.

A difference spectrum (reoxidized-resting) for a reoxidized cytochrome *c* oxidase sample that was initially reduced between 35 and 45% is shown in Fig. 3*a*. The peak at 604.2 nm may be assigned to the peroxidic adduct at the dioxygen reduction site, namely, Compound C by reference to earlier work (13). Similarly, the corresponding difference spectrum for a reoxidized sample that was initially completely reduced is shown in Fig. 3*b*. The peak at 601 nm in Fig. 3*b* is characteristic of the pulsed enzyme; a similar spectrum for the pulsed enzyme has been reported by Armstrong *et al.* (14). However, when a sample that was initially 85% reduced was reoxidized, the difference spectrum (Fig. 3*c*) revealed a number of novel spectral features (580 and 537 nm) that were not assignable to the peroxide adduct or the pulsed enzyme. A
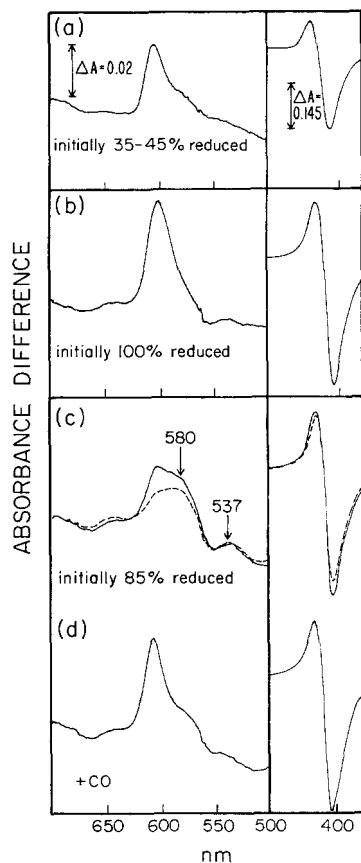

FIG. 3. **Difference spectra (reoxidized − resting) for cytochrome c oxidase samples with varying initial levels of reduction.** Difference spectra obtained after reoxidation of samples that were initially 35–45% (*a*) and 100% (*b*) reduced. The *solid difference spectrum* in *c* was obtained immediately after reoxidation of a sample that was initially 85% reduced. The *dashed difference spectrum* shown in *c* was obtained after 23 min of continued incubation at 277 K. When 1 atm of CO was admitted 28 min after reoxidation, the difference spectrum shown in *d* was obtained within 2–3 min (the earliest acquisition time). Spectra of the reoxidized samples were recorded within 2–3 min from the addition of O_2. Resting spectra were recorded before reduction of the samples.

broad trough centered at 668 nm was present in both the sample which was fully reduced and then reoxidized and the sample which was initially reduced by 85% and then reoxidized (Fig. 3, *b* and *c*).
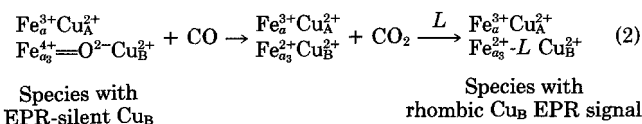
Assignments of the spectral features at 668, 580, and 537 nm has been aided by examining the reactivity of the sample toward CO. Addition of CO resulted within minutes (the time taken to record the spectrum through the 500 nm region) in the loss of the peak at 580 nm and a diminution of intensity of the 537 nm peak and a concomitant shift to 545 nm (Fig. 3*d*). The observed shift in the Soret from 435 to 437 nm after the addition of CO also suggests a reaction involving Fe_{a_3}, possibly the reduction of Fe_{a_3} from the ferryl to the ferrous state (Equation 2). There was no noticeable change in position or intensity of the 668 nm trough when CO was added to the sample; thus, this feature, which was also observed in the pulsed species produced by oxidation of the fully reduced enzyme, is not due to the EPR-silent precursor species.

Concomitant with the loss of intensity of the 580 and 537 nm peaks, we also observed increased intensity at 605.4 nm upon the addition of CO, which we ascribe to formation of the peroxidic adduct at the dioxygen reduction site, called Compound C (13) or the 607 nm complex (15). The latter species is expected when CO reacts, in the presence of dioxygen, with the pulsed subpopulation which is formed when fully reduced enzyme molecules are reoxidized (15, 16). The ferrous Fe_{a_3}-*L*/cupric Cu_B species, formed from the reaction of the three-electron reduced dioxygen species with CO, might also contribute to this intensity (10).

We have noted that the intermediate giving rise to the 580 and 537 nm peaks is quite stable in solution. On prolonged incubations, the intermediate decayed to the resting state. However, the half-time for this decay varied considerably from sample to sample, ranging from minutes to hours. This variability in stability is not surprising considering the varying amounts of reducing contaminants and denatured enzyme present in different samples.

DISCUSSION

Recent low temperature experiments from this laboratory showed that an EPR-silent, three-electron reduced, dioxygen intermediate was produced during reaction of reduced cytochrome *c* oxidase with dioxygen in solutions containing high concentrations of ethylene glycol and that this intermediate reacted at 211 K with an electron donor to yield a rhombic Cu_B EPR signal. Since CO was present as an inhibitor in these experiments, it was suggested to be the source of the electrons. The results reported here show that an EPR-silent, three electron-reduced, dioxygen intermediate also forms at room temperature, in the absence of ethylene glycol, and reacts rapidly with carbon monoxide at 275–298 K to yield a rhombic Cu_B EPR signal identical to that observed for the species formed at low temperatures, although in lower yields. Control experiments implicate CO as the source of electrons. Carbon monoxide is a two-electron donor. Because the dioxygen reduction site became reduced to only the ferrous/cupric state upon the addition of CO, while Cu_A and Fe_a remained oxidized, the intermediate that reacted with CO is by implication a ferryl Fe_{a_3} or a species at the same formal level of oxidation. Following Blair *et al.* (7) we propose that ferryl Fe_{a_3} reacts with CO according to Equation 2,

$$\begin{array}{c} Fe_a^{3+}Cu_A^{2+} \\ Fe_{a_3}^{4+}=O^{2-}Cu_B^{2+} \end{array} + CO \rightarrow \begin{array}{c} Fe_a^{3+}Cu_A^{2+} \\ Fe_{a_3}^{2+}Cu_B^{2+} \end{array} + CO_2 \xrightarrow{L} \begin{array}{c} Fe_a^{3+}Cu_A^{2+} \\ Fe_{a_3}^{2+}\text{-}L\ Cu_B^{2+} \end{array} \quad (2)$$

Species with
EPR-silent Cu_B

Species with
rhombic Cu_B EPR signal

where $L = O_2$ or CO.

If a sample of cytochrome c oxidase that is initially reduced between 50 and 100% is reoxidized there will be, at minimum, three subpopulations of intermediates initially present at the dioxygen reduction site: 1) Compound C, ii) a three-electron reduced species, and iii) the pulsed species. Upon the addition of CO to the reoxidized sample, all three subpopulations of intermediates can react with CO. Since CO is a two-electron donor, reduction by CO of the two subpopulations of even-electron intermediates, namely Compound C and pulsed, must result in products with integer spin, which are likely to be EPR-silent. However, when CO reacts with the three-electron reduced dioxygen intermediate another odd-electron intermediate must necessarily be produced. Thus, only the product of the reaction involving the odd-electron manifold of intermediates is visible by EPR.

The low yield of the rhombic Cu_B EPR signal in the present room temperature incubation experiments is understandable in the light of the three different subpopulations of intermediates present at the dioxygen reduction site after reoxidation of a partially reduced sample. Furthermore, the intermediate is expected to be short-lived in the EPR experiments because of the many cycles of freezing and thawing, which can contribute to enzyme denaturation and thus the production of endogenous reducing equivalents.

The nature of the EPR-silent, three-electron reduced, dioxygen intermediate may be more fully elucidated by comparing its optical spectrum with those enzymatic intermediates which are known to contain Fe(IV) centers. By comparison, the electronic absorption spectrum of the ferryl intermediate of cytochrome c peroxidase shows distinct α and β bands at 560 and 525 nm, respectively (17). According to the present study, the corresponding cytochrome c oxidase intermediate is characterized by two peaks at 580 and 537 nm in the optical spectrum, which we assign to the α and β bands, respectively, of ferryl Fe_{a_3}. The oxidase ferryl Fe_{a_3} α and β bands are expected to be red-shifted relative to the corresponding peaks for the ferryl (cytochrome c peroxidase) intermediate, as the heme in cytochrome c oxidase is heme A, whereas cytochrome c peroxidase contains protoporphyrin. These similarities of the optical spectrum of the three-electron reduced dioxygen species which we have trapped at room temperature to that of the ferryl cytochrome c peroxidase intermediate argues in favor of the proposal that the EPR-silent cytochrome c oxidase intermediate is also an Fe(IV) intermediate.

Wikström (18) has reported that when mitochondria are poised in a highly oxidized state with ferricyanide, the addition of ATP induces the formation of two intermediates which are similar, if not identical, to two of the intermediates that are formed when O_2 reacts with fully reduced cytochrome c oxidase. It was proposed that a partial reversal of the dioxygen reaction had occurred. The first intermediate Wikström observed upon stepwise reversal of the dioxygen reduction reaction was a 580 nm species. The second intermediate had intense absorption at 607 nm, making it spectroscopically similar to Compound C. Wikström suggested that the 580 nm species was a one-electron oxidation product of ferric Fe_{a_3}/cupric Cu_B, namely, a ferryl Fe_{a_3}/cupric Cu_B couple. In the light of our results, this conclusion appears correct; evidently the 580/537 nm species may be produced either by reducing dioxygen by three electrons or by reversing the O_2 reaction by a single electron transfer step.

Chance and co-workers (19, 20) have reported similar spectral features when hydrogen peroxide was added to pulsed cytochrome c oxidase. These workers proposed that the species giving rise to the spectral features at 537 and 580 nm was a peroxidic adduct at the dioxygen reduction site. In the light of the results presented here it is clear that the intermediate in question is not a peroxidic adduct. Additional experiments in support of this conclusion will be described elsewhere.

In conclusion, we have presented in this report chemical and spectroscopic evidence for the formation of a highly reactive ferryl Fe_{a_3} intermediate during turnover of cytochrome c oxidase. The present results strengthen our earlier suggestion that the ferryl-containing intermediate is produced at low temperature, and hence our suggestion that the dioxygen bond is broken at the formal three-electron level of reduction, in a highly activated but entropically favorable process (7). Further spectroscopic and chemical studies of the proposed ferryl Fe_{a_3} are presently in progress and these results will be presented in due course.

REFERENCES

1. Young, L., and Caughey, W. (1980) *Fed. Proc.* **39,** 2090 (Abstr. 2562)
2. Bickar, D. Bonaventura, C., and Bonaventura, J. (1984) *J. Biol. Chem.* **259,** 10777–10783
3. Brzezinski, P., and Malmström, B. G. (1985) *FEBS Lett.* **187,** 111–114
4. Morgan, J. E., Blair, D. F., and Chan, S. I., (1985) *J. Inorg. Biochem.* **23,** 295–302
5. Brudvig, G. W., Stevens, T. H., Morse, R. H., and Chan, S. I. (1981) *Biochemistry* **20,** 3912–3921
6. Armstrong, F., Shaw, R. W., and Beinert, H. (1983) *Biochim. Biophys. Acta* **722,** 61–71
7. Blair, D. F., Witt, S. N., and Chan, S. I. (1985) *J. Am. Chem. Soc.* **107,** 7389–7399
8. Hartzell, C. R., and Beinert, H. (1974) *Biochim. Biophys. Acta* **368,** 318–338
9. Reinhammar, B., Malkin, R., Jensen, P., Karlsson, B., Andréasson, L., Aasa, R., Vänngård, T., and Malmström, B., (1980) *J. Biol. Chem.* **255,** 5000–5003
10. Karlsson, B., and Andréasson, L. E. (1981) *Biochim. Biophys. Acta* **635,** 73–81
11. Blair, D. F., Martin, C. T., Gelles, J., Wang, H., Brudvig, G. W., Stevens, T. H., and Chan, S. I. (1983) *Chem. Scr.* **21,** 43–53
12. Aasa, R., and Vänngård, T. (1975) *J. Magn. Reson.* **19,** 308–315
13. Chance, B., and Leigh, J. S., Jr. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 4777–4780
14. Armstrong, F., Shaw, R. W., and Beinert, H. (1983) *Biochim. Biophys. Acta* **722,** 61–71
15. Nicholls, P. (1978) *Biochem. J.* **175,** 1147–1150
16. Nicholls, P., and Chanady, G. A. (1981) *Biochim. Biophys. Acta* **634,** 256–265
17. Ho, P. S., Hoffman, B. M., Kang, C. H., and Margoliash, E. (1983) *J. Biol. Chem.* **258,** 4356–4363
18. Wikström, M. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 4051–4054
19. Kumar, N., Naqui, A., and Chance, B. (1984) *J. Biol. Chem.* **259,** 11668–11671
20. Chance, B., Kumar, C., Powers, L., and Ching, Y. C. (1983) *Biophys. J.* **44,** 353–363