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Reactions of Nitric Oxide with Cytochrome *c* Oxidase†

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ABSTRACT: The reactions of nitric oxide (NO) with both oxidized and reduced cytochrome *c* oxidase are reported. NMR and mass spectroscopy were utilized to determine the products of the reactions; EPR and optical spectroscopy were employed to determine the states of the enzyme produced in each of these reactions. It was found that the enzyme catalyzes the consecutive oxidation and reduction of NO. A different cycle was observed when NO was added to the reduced enzyme, to the oxidized enzyme, or to the oxidized enzyme in the presence of azide. It was possible to observe the state of the enzyme at several points in each of these three cycles by varying the concentration of NO. The reactions of NO all involved a one- or two-electron redox step and could be accounted for by the involvement of only cytochrome a_3 and Cu_{a_3}. On the basis of these results, a mechanism for the reduction of dioxygen by the enzyme is proposed in which cytochrome a_3 functions to anchor dioxygen and intermediates while remaining in the ferrous state, whereas Cu_{a_3} functions to accept electrons from cytochrome a/Cu_a and transfer them to dioxygen.

Nitric oxide (NO) has been utilized extensively as a spin probe of the structure of oxygen binding proteins (Yonetani et al., 1972). The great utility of NO is that in addition to closely resembling dioxygen it also has one unpaired electron, which can be used to transform an even-spin site into an odd-spin site observable by EPR.[1] For example, ferroheme proteins, which normally do not exhibit any EPR signals, are transformed into a nitrosylferroheme species which exhibits a diagnostic EPR signal upon the addition of NO (Yonetani et al., 1972). In several other enzymes including cytochrome *c* oxidase (Malmström, 1979), hemocyanin (Lontie & Vanquickenborne, 1974), and laccase (Fee, 1975), two closely associated metal atoms form the oxygen binding sites, and a strong antiferromagnetic exchange interaction between the two metal centers renders the oxidized state of these enzymes EPR silent. Again if NO were to bind to one of the two metal centers of such an antiferromagnetically coupled pair, it could be possible to disrupt the antiferromagnetic coupling to produce a state observable by EPR spectroscopy as we recently demonstrated in our study of the interaction of NO with the oxygen binding site of oxidized cytochrome *c* oxidase (Stevens et al., 1979a).

In this paper, we have examined more closely the interactions of NO with cytochrome *c* oxidase. It will be shown that several reactions of NO are catalyzed by this enzyme, to both oxidize and reduce NO, and that these reactions can be utilized to produce states of the enzyme in which both cytochrome a_3 and Cu_{a_3} exhibit EPR signals. The elucidation of these re-

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[1] Abbreviations used: EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; PPD, *p*-phenylenediamine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; FT, Fourier transform; rf, radio frequency.

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actions as well as the nature of the states which are generated and observed by EPR in the course of the reactions has revealed new information on the structure of the oxygen binding site in this enzyme. Finally, the implications of these results for the mechanisms of oxygen binding and reduction are discussed.

Materials and Methods

Preparation of Cytochrome c Oxidase Complexes. Beef heart cytochrome *c* oxidase was isolated by the procedure of Hartzell & Beinert (1974). The purified protein was dissolved in 50 mM Tris–acetate buffer and 0.5% Tween 20, pH 7.4, and stored at −85 °C until use. The concentration of the enzyme was determined by the pyridine hemochromogen assay (Takemori & King, 1965), and the concentrations quoted in this paper were based on 2 molecules of heme *a*/enzyme. The preparations used in this work contained 9–11 nmol of heme *a*/mg of protein.

Nitric oxide (^{14}N), obtained from Matheson Coleman and Bell, is known to be contaminated with other nitrogen oxides and was therefore scrubbed with a dry ice/ethanol trap before being added to the enzyme samples. ^{15}NO (99.2% isotopic enrichment, Prochem) was found to be essentially free of other nitrogen oxides and was used as received. $K^{15}N^{14}N_2$ (99.4% isotopic enrichment), $Na^{15}NO_2$ (99.1% isotopic enrichment), and $K^{15}NO_3$ (99.1% isotopic enrichment) were all obtained from Prochem. All other reagents used were of the highest purity commercially available.

Four complexes of cytochrome *c* oxidase were prepared in this work: (1) oxidized enzyme–NO, (2) oxidized enzyme–azide–NO, (3) reduced enzyme–NO, and (4) reduced enzyme–nitrite. The samples were first made anaerobic by three cycles of evacuation and flushing with argon. Then NO was added to the samples through an inlet to the vacuum line so as to completely exclude oxygen. Denaturation of the protein (vide infra) was observed if strict anaerobic conditions were not maintained.

The oxidized enzyme–NO complex was prepared by the addition of NO to a pressure of 1 atm to an anaerobic sample of the oxidized enzyme in the absence of added ligands. The oxidized enzyme–azide–NO complex was prepared by adding NaN_3 to the oxidized enzyme to a concentration of 100 mM, making the sample anaerobic, and then adding NO to a pressure of 1 atm. The reduced enzyme–NO complex was prepared by adding the reductant (10 mM sodium ascorbate plus 5 mM PPD or 5 mM sodium dithionite) to the anaerobic oxidized protein from a side arm, incubating for 10 min to allow for complete reduction, and then adding NO to a pressure of 1 atm. The reduced enzyme–nitrite complex was prepared by adding 10 mM sodium ascorbate plus 5 mM PPD and 50 mM $NaNO_2$ to the anaerobic oxidized protein from a side arm. When ^{15}NO was added to the enzyme the same procedures were followed except that the pressure of ^{15}NO added to the samples was not measured but was ~1 atm.

Activity Assays. The activity of cytochrome *c* oxidase was measured polarographically with a YSI Model 53 oxygen electrode in a medium containing 50 mM phosphate buffer, 0.3 mg/mL cytochrome *c*, 1% Tween 80, and 30 mM ascorbate at pH 7.4 and 30.5 °C. The activity was measured before and after incubation of the oxidized enzyme for 24 h at 4 °C while mixing with an atmosphere of NO. In order to assay the activity of the enzyme which had been incubated with NO, it was necessary to extensively degas the sample, since exposure of the enzyme in the presence of NO to air resulted in denaturation of the enzyme. The NO was removed from the sample by five cycles of evacuation and flushing with argon. The activity was 114 ± 10 before NO incubation and 137 ± 20 after the incubation, in units of moles of cytochrome *c* oxidized per mole of cytochrome *c* oxidase per s. This control demonstrates that NO does not irreversibly alter the catalytic activity of the enzyme.

EPR Spectroscopy. EPR spectra were typically recorded on 0.2-mL samples containing the enzyme at a concentration of 0.25 mM. The exact concentration of the enzyme for each experiment is given in the figure legends. The EPR spectra were recorded on a Varian E-line century series X-band spectrometer equipped with an Air Products Heli-trans low-temperature system. All of our spectra were recorded between 10–20 K to achieve maximal resolution of the signals.

Optical Spectroscopy. The samples which were used for optical spectroscopy were prepared in a cuvette which was fitted with a ground-glass stopcock. We used 1 mm or 2 mm path length cells so that the high concentrations of the enzyme used in the EPR experiments could also be used to record optical spectra. All optical measurements were carried out at room temperature on a Beckman Acta CIII spectrometer.

Mass Spectroscopy. The samples for mass spectroscopy were prepared in a 5-mL glass bulb which was fitted with a ground-glass stopcock. A 1.0-mL aliquot of cytochrome *c* oxidase was used in each experiment. After the addition of NO, a magnetic stir bar inside the bulb was used to mix the sample with the NO atmosphere. The reaction was then allowed to proceed for the specified length of time, and, thereafter, the gas above the sample was fed directly through a ground-glass inlet into a Du Pont 21-492B mass spectrometer. With this procedure, only the gaseous nitrogen compounds NH_3, N_2, NO, and N_2O could be monitored, since the other gaseous nitrogen compounds, in particular NO_2, are not stable at room temperature in the presence of water. In these experiments, atmospheric CO_2 and N_2 were the major contaminants and interferred with the observation of the N_2O and N_2 parent peaks. ^{15}NO was substituted for ^{14}NO and/or $^{15}N^{14}N_2^-$ was substituted for $^{14}N_3^-$ to alleviate this problem. The parent peaks due to $^{15}N_2O$, $^{14}N^{15}NO$, $^{15}N_2$, and $^{15}N^{14}N$ could be observed without interference. In each experiment, a blank was also prepared which was identical with the sample except that the enzyme was omitted. All mass spectral data quoted in this work were corrected for the background observed in the blank.

NMR Spectroscopy. The samples, which were used for NMR spectroscopy, were prepared in a 10-mm NMR tube which was constricted near the top and fitted with a ground-glass stopcock. In order to minimize the effect of sample vortexing, we used 4-mL samples. The samples contained 25% D_2O, which was used for an internal lock, and the enzyme concentration was 0.12 mM. After ^{15}NO was added to the anaerobic samples to a pressure of ~1 atm, the sample plus ^{15}NO gas was frozen in liquid nitrogen and the NMR tube was sealed and removed from the stopcock at the constriction. Then the sample was thawed and placed on a motor-driven carrousel at 4 °C which repeatedly inverted the sample to provide mixing of the solution with the NO atmosphere. After the desired period of incubation, the ^{15}N NMR spectrum of the solution was recorded at 30 °C on a JEOL FX90 NMR spectrometer.

These NMR spectroscopy experiments are complementary to the mass spectroscopy experiments described in the previous section in that gaseous reaction products were observed by mass spectroscopy and nongaseous products were observed by NMR. However, the sensitivity of ^{15}N NMR is considerably lower than that of mass spectroscopy, and a product concen-

Table I: EPR Signals Observed for Cytochrome *c* Oxidase and Its NO Complexes

complex	species present	EPR signals (*g* values)
oxidized enzyme[a] or oxidized "oxygenated" enzyme plus NO[b]	ferricytochrome *a*	3.03, 2.21, 1.45
	oxidized Cu_a center	2.18, 2.03, 1.99
	ferricytochrome $a_3/Cu_{a_3}^{2+}$	EPR silent
oxidized enzyme plus NO[b]	ferricytochrome *a*	3.03, 2.21, 1.45
	oxidized Cu_a center	2.18, 2.03, 1.99
	ferricytochrome a_3	6.16, 5.82, 2.0
	$Cu_{a_3}^{2+}$-NO	EPR silent
oxidized enzyme plus N_3^- plus NO[b]	ferricytochrome *a*	3.03, 2.21, 1.45
	oxidized Cu_a center	2.18, 2.03, 1.99
	nitrosylferrocytochrome $a_3/Cu_{a_3}^{2+}$	triplet signal; main transition from $g = 2.61$ to $g = 1.69$, half-field transition at $g = 4.3$
reduced enzyme[a]	ferrocytochrome *a*	EPR silent
	reduced Cu_a center	EPR silent
	ferrocytochrome $a_3/Cu_{a_3}^{+}$	EPR silent
reduced enzyme plus limited NO[c]	ferrocytochrome *a*	EPR silent
	reduced Cu_a center	EPR silent
	$Cu_{a_3}^{+}$	EPR silent
	nitrosylferrocytochrome a_3	2.09, 2.00, 1.97 (9 line hyperfine structure on $g = 2.00$)
reduced enzyme plus excess NO[d]	ferrocytochrome *a*	EPR silent
	reduced Cu_a center	EPR silent
	nitrosylferrocytochrome $a_3/Cu_{a_3}^{+}$-NO	EPR silent
NO dissolved in buffer[b]	NO	1.97, 1.97, 1.7

[a] Aasa et al., 1976. [b] Stevens et al., 1979a. [c] Blokzijl-Homan & Van Gelder, 1971. [d] This work.

tration of ~5 mM or more was required before the NMR signal of a reaction product could be observed. For this reason much longer reaction times were required for the NMR experiments (generally several days at 4 °C).

Results

In the presence of NO, EPR signals can be observed from all four metal centers in cytochrome *c* oxidase, depending on the oxidation and/or ligation state of the enzyme. We have summarized in Table I the states of the enzyme which have previously been observed in the presence of NO plus those which we will describe in this work. Cytochrome *a* and Cu_a exhibit EPR signals in the oxidized state which are eliminated upon reduction of these sites. Neither of these metal centers interact directly with NO. It is not quite as straightforward to determine the oxidation and ligation states of cytochrome a_3 and Cu_{a_3} by EPR spectroscopy. In the oxidized enzyme, these two metal centers are strongly antiferromagnetically coupled (Tweedle et al., 1978), while, in the reduced enzyme, no EPR signals are expected from the high-spin cytochrome a_3. However, in the presence of NO, three states do exhibit diagnostic EPR signals: (1) ferricytochrome $a_3/Cu_{a_3}^{2+}$-NO (high-spin cytochrome a_3 EPR signal), (2) nitrosylferrocytochrome $a_3/Cu_{a_3}^{2+}$ (triplet EPR signal), and (3) nitrosylferrocytochrome $a_3/Cu_{a_3}^{+}$ (nitrosylferrocytochrome a_3 EPR signal).

In this work, we utilized EPR spectroscopy to determine the states of the metal centers which are formed during the reactions with NO. As we will demonstrate, NO can both oxidize and reduce cytochrome *c* oxidase. Under the appropriate conditions, a cycle was established in which the oxidation state of the enzyme oscillated as NO was consecutively oxidized and reduced. It was possible to follow these reaction cycles by EPR only because the rate of consumption of NO was quite slow. This allowed us to initiate the reaction by adding NO, incubate the sample for a specified length of time at 20 °C, quickly freeze the sample by placing it in the Air Products Heli-trans Dewar at 10 K to trap the states present, and then monitor the state of the enzyme by EPR. At each instant in time, the major steady-state species observed should be the one which just preceded the slowest step in the reaction cycle. Depending on the concentration of the dissolved NO, this slowest step in the reaction cycle could be either a redox reaction, if excess NO was present, or the binding of NO to the enzyme, if the NO was limited. It was possible to vary the NO concentration by incubating the sample without mixing, since the rate at which NO diffused from the gas above the sample into the unstirred solution was insignificant compared to the rate at which NO was consumed by the reactions. By this procedure, we, therefore, observed the steady-state species as a function of NO concentration.

Reduced Cytochrome c Oxidase plus NO. (1) Dithionite as Reductant. In most of the previous work done on the nitrosyl derivative of reduced cytochrome *c* oxidase, dithionite was used to reduce the enzyme (Blokzijl-Homan & Van Gelder, 1971; Stevens et al., 1979b). We also initially employed dithionite to reduce the enzyme. However, we have noted that the enzyme was unstable in the presence of both dithionite and NO as the enzyme precipitated within minutes after the addition of NO. Moreover, when NO was added to the enzyme reduced by dithionite, no EPR signal was observed from the NO dissolved in the buffer, even when the sample was mixed and rapidly frozen; only the EPR signal from nitrosylferrocytochrome a_3 was observed. This observation demonstrates that a reaction was occurring which reduced the concentration of dissolved NO to a level which was too small to be detected by EPR.

In order to determine what reaction took place to consume the NO, we examined the mass spectrum of the products formed from the incubation of 100 mM sodium dithionite in 50 mM Tris–acetate, pH 7.4, under an NO atmosphere at room temperature. It was found that essentially all of the NO was converted into N_2O within 20 min. We followed the rate of NO consumption by dithionite manometrically (after correcting for the N_2O evolved). The results (Figure 1) show that 2 mol of NO was rapidly consumed per mol of dithionite, and, thereafter, a slower reaction continued to consume NO. In addition, we have noted a change in pH of the sample resulted from the reaction of NO with dithionite. In the presence of excess NO, the pH dropped from 5.8 (the pH of a solution of 50 mM Tris–acetate buffered to pH 7.4 plus 0.5 M sodium dithionite) to 2.2 after incubation of the sample for 24 h at room temperature. In contrast, when the mole ratio of NO to dithionite was equal to 1, the pH of the solution dropped only from 5.8 to 5.6.

All of the above can be explained by reactions 1 and 2, provided that reaction 1 is much more rapid than reaction 2.

$$S_2O_4^{2-} + 2NO + H_2O \rightarrow 2HSO_3^- + N_2O \quad (1)$$

$$HSO_3^- + 2NO \rightarrow SO_4^{2-} + N_2O + H^+ \quad (2)$$

The first reaction should not lower the pH much below pH 6, since the pK_a of HSO_3^- is 6.9. However, reaction 2 would decrease the pH significantly below pH 6, since the pK_a of HSO_4^- is 1.9. These reactions account for the instability of cytochrome *c* oxidase in the presence of dithionite and NO. Since dithionite is the limiting reagent in most reduction studies on cytochrome *c* oxidase, its rapid reaction with NO would produce a large pH drop in the sample, which then causes the enzyme to precipitate. Therefore, NO-binding studies involving dithionite as the reducing agent should not be carried out unless the sample is strongly buffered.

(*2*) *Ascorbate plus PPD as Reductant.* Ascorbate with PPD as the mediator can also be used to reduce cytochrome *c* oxidase. In order to ascertain whether the ascorbate plus PPD reduction of the enzyme was complicated by side reactions such as those with dithionite, we first examined the rate of reaction of ascorbate plus PPD with NO. The mass spectra of the products formed after the reaction of ^{15}NO with 10 mM ascorbate plus 5 mM PPD in 50 mM Tris–acetate buffer, pH 7.4, in the presence and absence of cytochrome *c* oxidase are shown in Figure 2. Even after 11.5 h of incubation, the reaction of ascorbate plus PPD with NO was not pronounced; however, a small amount of N_2O was formed. $^{15}N_2O$ was produced when ^{15}NO was used and $^{14}N_2O$ was produced when ^{14}NO was used. The production of N_2O is probably due to the slow reduction of NO to N_2O by ascorbate (reaction 3).

$$\text{ascorbate} + 2NO \rightarrow \text{dehydroascorbate} + N_2O + H_2O \quad (3)$$

In the presence of cytochrome *c* oxidase, however, the amount of N_2O produced was twofold greater. Therefore, reaction 3 appears to be catalyzed by cytochrome *c* oxidase.

When NO was added to cytochrome *c* oxidase which was reduced by *dithionite*, the enzyme exhibited a nitrosylferrocytochrome a_3 EPR signal and no EPR signal from the NO dissolved in the buffer. In contrast, when NO was added to the enzyme which was reduced by *ascorbate plus PPD*, we observed a large EPR signal from the NO dissolved in the buffer but essentially no EPR signal from the enzyme (Figure 3A). However, when this sample was then allowed to incubate without mixing at room temperature, the NO dissolved in the buffer was consumed, as evidenced by the gradual disappearance of the NO EPR signal at $g = 1.97$. As the concentration of NO in solution decreased, the nitrosylferrocytochrome a_3 EPR signal increased in intensity (Figure 3B). The EPR signal observed from nitrosylferrocytochrome a_3 prepared in this manner was identical with that observed when NO was added to the enzyme which was reduced with dithionite. Remixing the sample restored the large NO EPR signal at $g = 1.97$ and eliminated the nitrosylferrocytochrome a_3 EPR signal. As long as both excess NO and excess reducing agent were available, the cycle could be repeated. However, when NO was in excess over the amount of ascorbate plus PPD and the reductant was ultimately completely consumed, the enzyme exhibited an EPR signal identical with that of the native oxidized enzyme, indicating that cytochrome *a* and Cu_a became reoxidized.

We examined the optical spectrum of cytochrome *c* oxidase reduced with ascorbate plus PPD in the presence of NO. The optical spectrum of the reduced enzyme which had just been mixed with NO is shown in Figure 4. The spectrum exhibited a characteristic split Soret peak with maxima at 441 nm, due to ferrocytochrome *a*, and at 428 nm, due to nitrosylferrocytochrome a_3 (Stevens et al., 1979a). However, incubation of this sample until the dissolved NO was used up (a procedure

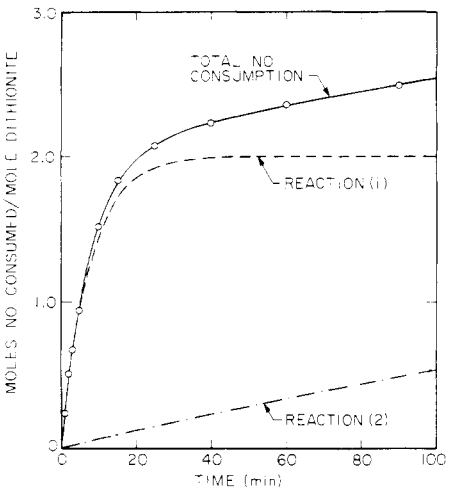

FIGURE 1: Manometric measurement of NO consumption when excess NO was added to an anaerobic solution of 100 mM sodium dithionite and 50 mM Tris–acetate at pH 7.0. A 3.0-mL solution was stirred constantly and maintained at 30.0 °C by a temperature-regulated water bath. The consumption of NO was noted to be biphasic, and the data were fitted to the sum of two exponentials: (1) a rapid reaction which consumed 2 mol of NO/mol of dithionite, which has been attributed to reaction 1, and (2) a slow reaction, which has been attributed to reaction 2. See text for details.

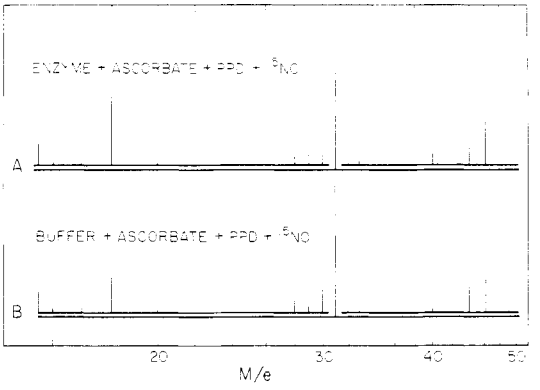

FIGURE 2: Mass spectra of (A) the gaseous compounds obtained after 1.0 mL of 80 μM cytochrome *c* oxidase plus 60 mM ascorbate and 5 mM PPD was incubated with ^{15}NO for 11.5 h and (B) the gaseous compounds obtained in a sample identical with that in A except without the enzyme. The ^{15}NO parent peaks ($m/e = 31$) are attenuated 10-fold from the rest of the peaks.

which yields the nitrosylferrocytochrome a_3 EPR signal only) did not alter the optical spectrum from that shown in Figure 4. As in our EPR studies, we were able to observe the reoxidation of the enzyme optically after prolonged mixing of the sample with NO (Figure 4). However, the optical spectrum of the enzyme reoxidized by NO was not characteristic of the fully oxidized enzyme, but rather a mixture of fully oxidized molecules with molecules in which cytochrome *a* was oxidized and cytochrome a_3 reduced with NO bound. This point will be elaborated on in a later section in which we discuss the interaction of NO with the oxidized enzyme.

Our EPR results showed that one NO binds to cytochrome a_3 tightly; even when the concentration of NO in solution was too small to be detected by EPR, the nitrosylferrocytochrome a_3 EPR signal was observed. However, when the concentration of dissolved NO was increased, the nitrosylferrocytochrome a_3 EPR signal disappeared, although the optical spectrum of the reduced enzyme in the presence of excess NO indicated that NO remained bound to ferrocytochrome a_3. In order to eliminate the EPR signal from nitrosylferrocytochrome a_3 without changing the oxidation or ligation state of cytochrome

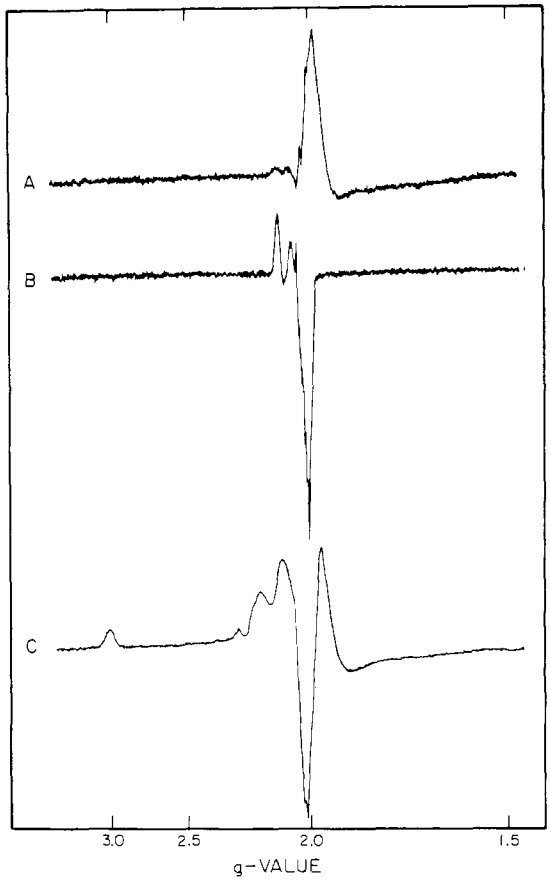

FIGURE 3: EPR spectra of (A) 0.20 mM cytochrome *c* oxidase reduced with 1.5 mM ascorbate and 1.5 mM PPD, then mixed with 1-atm NO, and rapidly frozen; (B) sample A incubated for 2 h at 20 °C without mixing; (C) sample A incubated 22 h at 4 °C while mixing. The conditions were temperature, 15 K; microwave power, 0.05 mW (A and B) and 0.2 mW (C); modulation amplitude, 5 G (A and B) and 16 G (C); and microwave frequency, 9.25 GHz.

a_3, it is necessary to magnetically couple another paramagnetic site to the nitrosylferrocytochrome a_3 site. If Cu_{a_3} were oxidized, the site should exhibit a triplet signal as is observed when NO and azide are added to the oxidized enzyme (Table I). Therefore, we propose that a second NO was bound to Cu_{a_3} at higher NO concentrations and presumably was magnetically coupled to the nitrosylferrocytochrome a_3 site such that the enzyme no longer exhibited an EPR signal. As a further check, we examined the effect of nitrite and nitrous oxide (possible reaction products of NO) on the nitrosylferrocytochrome a_3 EPR signal and found no evidence that either of these molecules interact with the reduced NO-bound enzyme.

When two NO molecules are bound, the enzyme can donate two electrons to reduce two NO molecules according to reaction 4. In this manner, the cytochrome *c* oxidase catalyzed

$$2NO + 2e^-(\text{enzyme}) + 2H^+ \rightarrow N_2O + H_2O \quad (4)$$

reduction of NO may be understood.

It, therefore, seems that a cycle is established when NO is added to the reduced enzyme, with ascorbate plus PPD providing the enzyme with two electrons which the enzyme then uses to reduce NO. This cycle is schematically shown in Figure 5. State A is the fully reduced enzyme; state B is formed when the first NO binds to the enzyme and exhibits the nitrosylferrocytochrome a_3 EPR signal; state C has two NO molecules bound and is EPR silent; state D is the transient species formed when two electrons are transferred to NO. We have not observed state D directly. It is probable that electron

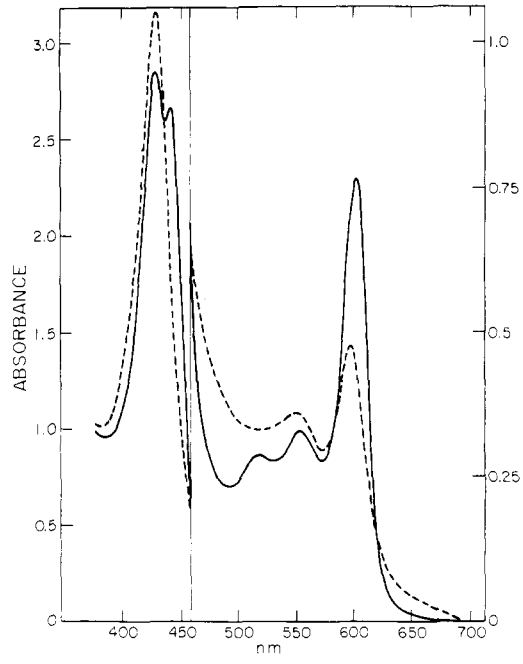

FIGURE 4: Optical spectra of 90 μM cytochrome *c* oxidase reduced with 1.0 mM ascorbate plus 0.5 mM PPD immediately after mixing with 1-atm NO (solid line) and after incubation at 4 °C for 24 h while mixing until a steady state was achieved (dashed line). The spectra were recorded in a 2 mm path length cell at 20 °C.

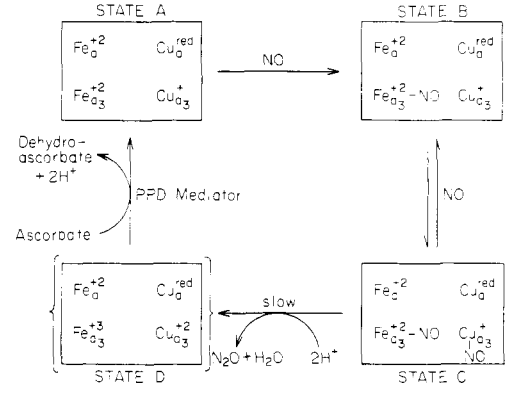

FIGURE 5: The proposed reaction cycle which occurs when cytochrome *c* oxidase is incubated with NO in the presence of ascorbate and PPD. In this cycle cytochrome *a* and Cu_a remain reduced except possibly during electron redistribution in the transient state D. An oxidation state has not been assigned to Cu_a itself, but rather to the Cu_a center, in view of the possibility that Cu_a remains in the cuprous state in both the oxidized and reduced enzyme while an associated sulfur ligand accepts electrons [see Chan et al. (1979) for additional discussion].

redistribution within the enzyme occurs rapidly. Thereafter, the binding of NO to cytochrome a_3 and the full reduction of the enzyme by ascorbate plus PPD would occur. Since we do not observe any EPR signals from the enzyme in the presence of excess NO, the rate-determining step in the overall cycle must be the step between states C and D.

Oxidized Cytochrome c Oxidase plus N_3^- plus NO. In our earlier work, it was shown that when NO is added to oxidized cytochrome *c* oxidase in the presence of azide, cytochrome a_3 is reduced and a triplet species is formed from the interaction of nitrosylferrocytochrome a_3 ($S = {}^1/_2$) with $Cu_{a_3}^{2+}$ ($S = {}^1/_2$) (Stevens et al., 1979a). In this earlier work, it was concluded that the reduction of cytochrome a_3 occurred via reaction 5.

$$\text{ferricytochrome } a_3 + NO + N_3^- \rightarrow \text{ferrocytochrome } a_3 + N_2O + N_2 \quad (5)$$

This conclusion was made on the basis of an analysis of the

Table II: Summary of Mass Spectral Data: Comparison of the Isotopic Distribution among N_2 and N_2O Parent Peaks Observed with That Predicted on the Basis of the Reaction Cycles Discussed in the Text

	$^{14}N_2$		$^{15}N^{14}N$		$^{15}N_2$		$^{14}N_2O$		$^{14}N^{15}NO$ plus $^{15}N^{14}NO$		$^{15}N_2O$	
	obsd	pred	obsd	pred	obsd	pred	obsd	pred	obsd	pred	obsd	pred
reduced cytochrome *c* oxidase												
(a) plus ^{14}NO	0	0	0	0	0	0	1.00	1.00	0	0	0	0
(b) plus ^{15}NO	0	0	0	0	0	0	0	0	0	0	1.00	1.00
oxidized cytochrome *c* oxidase												
(a) plus $^{14}N_3^- + {}^{14}NO$	1.00	1.00	0	0		0	1.00	1.00	0	0	0	0
(b) plus $^{14}N_3^- + {}^{15}NO$	0.96	1.00	0.01	0	0.03	0	0.02	0	0.47	0.50	0.51	0.50
(c) plus $^{15}N^{14}N^{14}N^- + {}^{14}NO$	0.53	0.5	0.47	0.5		0	0.69	0.75	0.31	0.25	0	0
(d) plus $^{15}N^{14}N^{14}N^- + {}^{15}NO$	0.37	0.5	0.54	0.5	0.09	0	0.04	0	0.24	0.25	0.72	0.75

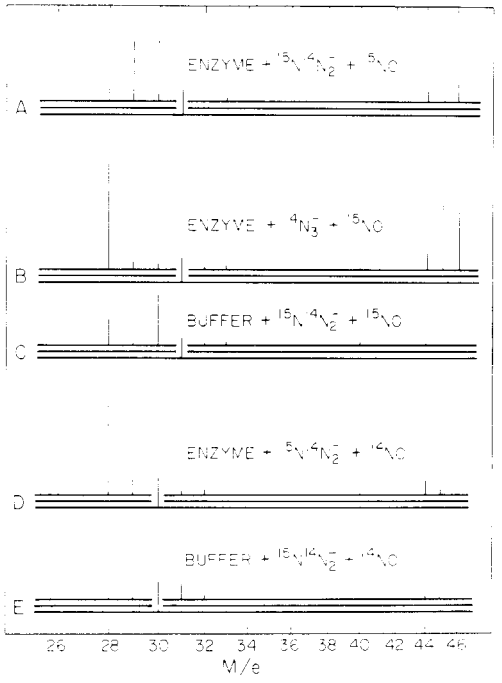

FIGURE 6: Mass spectra of the gaseous compounds obtained after 10 h of incubation at 20 °C of 1.0-mL samples of the oxidized enzyme with azide and NO. Each sample contained 100 mM azide and the enzyme concentration was 0.25 mM. The background spectra were identical with spectra of the NO gas alone and representative spectra are shown in C for ^{15}NO and E for ^{14}NO. The NO parent peaks are attenuated 100-fold from the rest of the peaks.

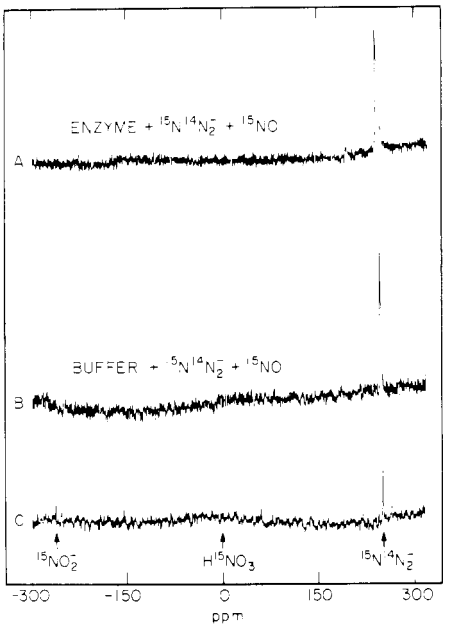

FIGURE 7: ^{15}N FT NMR spectra of (A) 0.12 mM oxidized cytochrome *c* oxidase plus 100 mM $Na^{15}N^{14}N_2$ and ~1-atm ^{15}NO incubated 48 h at 4 °C; (B) a sample identical with that in A except without the enzyme; (C) an aqueous solution of 10 mM $Na^{15}NO_2$ and 100 mM $Na^{15}N^{14}N_2$ with an insert containing a 70% solution of HNO_3. The conditions were temperature, 30 °C; rf, 9.04 MHz; pulse angle, 60°; acquisition time, 0.682 s; spectral width, 6000 Hz; and transients, 70 000 (A), 72 000 (B), 21 000 (C).

reaction products by mass spectroscopy.

We have now examined this reaction in greater detail by the use of both ^{15}NO and $^{15}N^{14}N_2^-$ isotopic labeling. In particular, we have made a quantitative analysis of the isotopic labeling in the N_2O and N_2 reaction products. These results are shown in Figure 6 and summarized in Table II.

On the basis of reaction 5, we would expect that the NO nitrogen along with one of the outer azide nitrogens would be incorporated into the N_2O produced and the remaining two azide nitrogens to be incorporated into dinitrogen. If only this reaction occurred, then the use of ^{15}NO and $^{14}N_3^-$ should produce only $^{14}N^{15}NO$ and $^{14}N_2$. However, as seen in Figure 6B, we observed the production of $^{15}N_2O$ in equal amounts to $^{14}N^{15}NO$, while $^{14}N_2$ was the only dinitrogen product. This interesting observation can be explained if reaction 5 occurred the same number of times as reaction 4. Furthermore, the use of isotopically labeled NO in combination with labeled azide gave the intensity ratios of labeled products expected if reactions 4 and 5 each occur an equal number of times (Table II).

It is evident from the above results that, in the presence of azide, NO can first reduce and then oxidize the enzyme. However, reaction 5 provides only one electron while reaction 4 consumes two electrons. Another one-electron step which does not generate any gaseous products must occur to balance the overall reaction. To resolve this point, we examined the ^{15}N NMR spectrum of the reaction products formed in the reaction of ^{15}NO and $^{15}N^{14}N_2^-$ with the oxidized enzyme. The results are shown in Figure 7. After 48 h of incubation at 4 °C, we observed a peak 509.8 ppm downfield from $^{15}N^{14}N_2^-$ which can be assigned to nitrite. This peak was only observed in the sample containing the enzyme. Thus, nitrite was formed as part of the reaction cycle, suggesting reaction 6. The cycle

$$Cu_{a_3}^{2+} + NO + H_2O \rightarrow Cu_{a_3}^{+} + NO_2^- + 2H^+ \quad (6)$$

formed then was (1) the reduction of cytochrome a_3 via reaction 5, (2) the reduction of Cu_{a_3} via reaction 6, and (3) the oxidation of both Cu_{a_3} and cytochrome a_3 via reaction 4.

The above conclusions were verified by examination of the EPR spectra of cytochrome *c* oxidase samples which were allowed to incubate at room temperature in the presence of azide and NO for variable lengths of time. The results are shown in Figure 8. We observed the triplet EPR signal characteristic of nitrosylferrocytochrome $a_3/Cu_{a_3}^{2+}$, in the presence of excess NO (Figure 8A). When the sample was

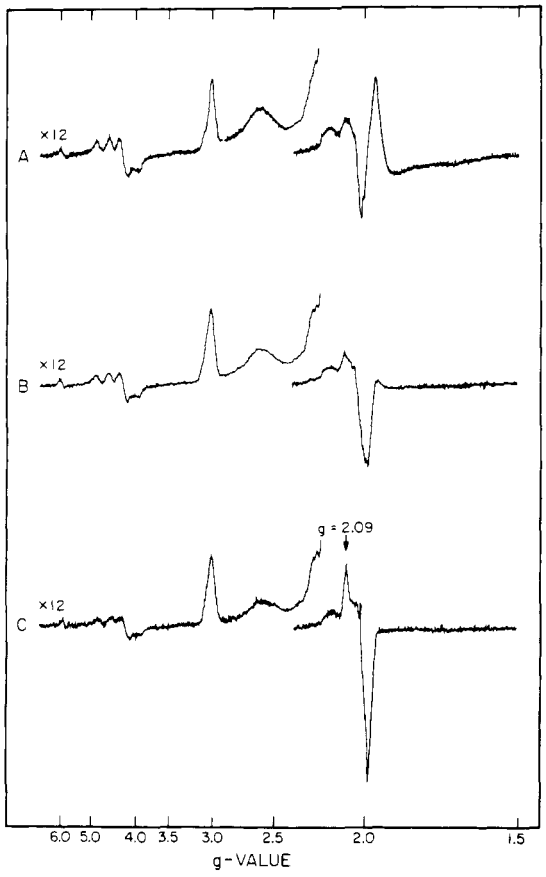

FIGURE 8: EPR spectra of (A) 0.25 mM oxidized cytochrome *c* oxidase plus 100 mM azide and 1-atm NO which was mixed, incubated 10 min, remixed, and rapidly frozen; (B) sample A incubated 30 min at 20 °C without mixing; (C) sample A incubated a total of 60 min at 20 °C without mixing. The conditions were temperature, 15 K; microwave power, 0.05 mW; modulation amplitude, 5 G; and microwave frequency, 9.25 GHz. The low-field portions of the spectra were recorded with a 4-fold higher modulation amplitude and a 9-fold higher microwave power to give a 12-fold greater intensity compared to the high-field portion.

then allowed to incubate at room temperature, the NO dissolved in the buffer was slowly consumed as evidenced by the disappearance of the signal at $g = 1.97$. Concomitant with the disappearance of the dissolved NO was the appearance of a nitrosylferrocytochrome a_3 EPR signal with its characteristic sharp maximum at $g = 2.09$ indicating the reduction of Cu_{a_3}. As the nitrosylferrocytochrome a_3 EPR signal increased in intensity, the nitrosylferrocytochrome $a_3/Cu_{a_3}^{2+}$ triplet EPR signal intensity decreased. The triplet EPR signal, however, never completely disappeared, even after prolonged incubation of the sample at room temperature to reduce the concentration of dissolved NO to a level no longer detectable by EPR. Again, remixing the sample with NO restored the spectrum shown in Figure 8A (remixed spectrum not shown).

The above results suggest the reaction cycle depicted in Figure 9 when azide and NO are added to oxidized cytochrome *c* oxidase. Here state E refers to the oxidized enzyme. One-electron reduction of the oxidized enzyme via reaction 5 produced state F, the nitrosylferrocytochrome $a_3/Cu_{a_3}^{2+}$ state, which exhibits the triplet EPR signal. Then Cu_{a_3} was reduced via reaction 6 to form state G, the nitrosylferrocytochrome $a_3/Cu_{a_3}^{+}$ state, which exhibits the nitrosylferrocytochrome a_3 EPR signal. The binding of a second NO to the two-electron reduced enzyme formed state H in which cytochrome a_3/Cu_{a_3} are EPR silent, and finally the reoxidation of the enzyme occurred via reaction 4.

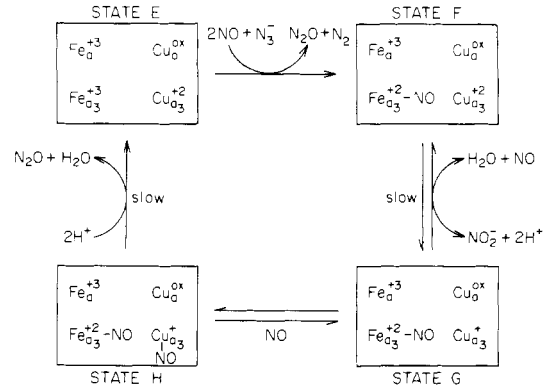

FIGURE 9: The proposed reaction cycle which occurs when oxidized cytochrome *c* oxidase is incubated with azide and NO.

It is evident that, in the presence of excess NO, the reduction of Cu_{a_3} is the rate-limiting step. However, when the concentration of dissolved NO was sufficiently low, the rate at which NO binds to the two-electron reduced enzyme becomes comparable to the rate of reduction of Cu_{a_3}. Accordingly, a steady state is established with comparable concentrations of the nitrosylferrocytochrome $a_3/Cu_{a_3}^{2+}$ and nitrosylferrocytochrome $a_3/Cu_{a_3}^{+}$ species. Note that in the reaction cycle, states G and H are analogous to states B and C, respectively, in Figure 5. It should also be noted that throughout this reaction cycle both cytochrome *a* and the Cu_a center remained fully oxidized and were observed in the EPR spectrum.

Reduced Cytochrome c Oxidase plus NO_2^-. In the previous section it was noted that NO can reduce Cu_{a_3} with the formation of nitrite (reaction 6). On the basis of the free energy associated with this reaction under standard conditions, we expect excess nitrite to react with reduced cytochrome *c* oxidase to form NO and, in the presence of excess reductant, the nitrosylferrocytochrome $a_3/Cu_{a_3}^{+}$ state. We found that nitrite did react with cytochrome *c* oxidase reduced with ascorbate and PPD (reaction 7) to form the nitrosylferrocytochrome $a_3/Cu_{a_3}^{+}$ state. This state of the enzyme exhibited

$$NO_2^- + e^-(\text{enzyme}) + 2H^+ \rightarrow NO + H_2O \qquad (7)$$

an EPR signal from nitrosylferrocytochrome a_3 which was identical with that observed when NO was added to the dithionite-reduced enzyme or when limited NO was added to the enzyme reduced by ascorbate plus PPD.[2]

Yonetani et al. (1972) previously utilized the reaction of dithionite plus nitrite to generate NO in their preparation of nitrosylferroheme complexes. It is possible that ascorbate and PPD also react with nitrite to produce free NO. However, this reaction is quite slow. It was found that no nitrosylferrocytochrome *c* EPR signal was generated when nitrite plus ascorbate and PPD were added to cytochrome *c*, even after 4 h of incubation at room temperature. In addition, no EPR signal from NO dissolved in the buffer was observed in this sample. These experiments demonstrate that in the case of cytochrome *c* oxidase the reduction of nitrite to NO occurred at the oxygen binding site of the enzyme.

Oxidized Cytochrome c Oxidase plus NO. When NO is added to oxidized cytochrome *c* oxidase a high-spin cytochrome a_3 EPR signal is observed. The formation of this complex was discussed by Stevens et al. (1979a), and it was proposed that

[2] The formation of the nitrosylferrocytochrome $a_3/Cu_{a_3}^{+}$ complex by this method does not require the use of either NO gas or dithionite. The use of ascorbate plus PPD to reduce the enzyme followed by the addition of nitrite appears to be the simplest method to form this enzyme complex.

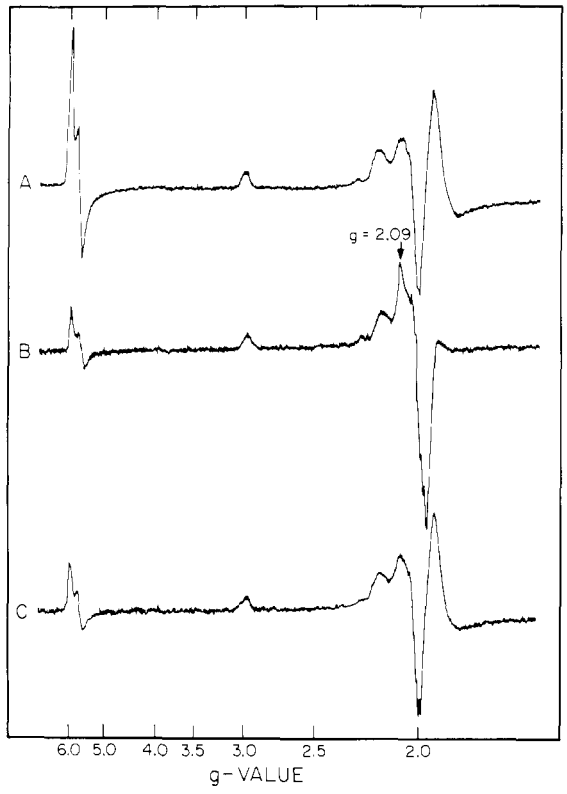

FIGURE 10: EPR spectra of (A) 0.25 mM oxidized cytochrome *c* oxidase plus 1-atm NO which was mixed and rapidly frozen; (B) sample A incubated at 20 °C without mixing for 8 h; (C) sample B remixed with NO and rapidly frozen. The EPR spectrum obtained after rapid removal of NO and immediate freezing of sample C exhibited a signal at $g = 2.09$ of the same intensity as in B. The conditions were temperature, 11 K; microwave power, 0.02 mW; modulation amplitude, 10 G; and microwave frequency, 9.25 GHz.

NO binds to Cu_{a_3} and breaks the antiferromagnetic coupling between Cu_{a_3} and cytochrome a_3. Although for short incubation times, NO binds reversibly to the oxidized enzyme, subsequent work has shown that prolonged incubation of the oxidized enzyme with NO leads to reduction of cytochrome a_3 and Cu_{a_3}.

The EPR spectrum of the oxidized enzyme which has been mixed with NO and immediately frozen is shown in Figure 10A. A high-spin cytochrome a_3 EPR signal at $g = 6$ was observed in addition to the cytochrome a, Cu_a, and NO EPR signals. When the sample was incubated at 20 °C without mixing, the NO in solution was slowly consumed. Paralleling the consumption of NO was a decrease in intensity of the high-spin cytochrome a_3 EPR signal. Finally the nitrosylferrocytochrome a_3 EPR signal appeared when the concentration of NO in solution was lowered significantly (Figure 10B). While remixing the sample with NO eliminated the nitrosylferrocytochrome a_3 EPR signal, it did not restore the high-spin cytochrome a_3 EPR signal (Figure 10C). Rapid removal of NO from the sample at this point, however, restored the nitrosylferrocytochrome a_3 EPR signal.

The intensity of the nitrosylferrocytochrome a_3 EPR signal after removal of the NO from the sample is a measure of the fraction of molecules in which both cytochrome a_3 and Cu_{a_3} are reduced. Thus, the time course of the reduction of these metal centers can be followed by first incubating the sample with NO for a specified length of time, then rapidly removing the NO from the sample, and finally freezing the sample to monitor the EPR spectrum. The intensity of the nitrosylferrocytochrome a_3 EPR was observed to increase with the time of incubation with NO. However, in the steady state,

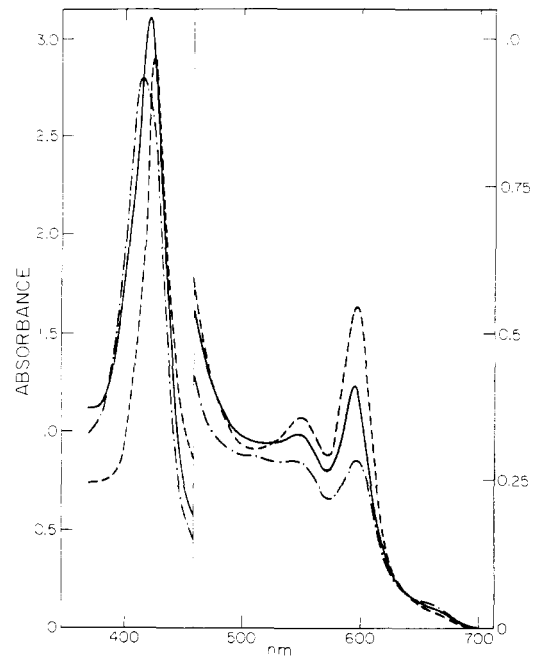

FIGURE 11: Optical spectra of 90 μM oxidized cytochrome *c* oxidase plus 1-atm NO which was mixed and immediately recorded (-·-) and incubated at 4 °C for 24 h while mixing until a steady state was achieved (—). For comparison, the spectrum of the one-quarter reduced NO-bound enzyme is also shown (---). The one-quarter reduced enzyme was prepared by the reaction of azide and NO with the oxidized enzyme. The spectra were recorded in a 2 mm path length cell at 20 °C.

which was reached after ~10 h of incubation at room temperature, only about 10–30% of the enzyme molecules were in a state which exhibited this EPR signal. This fraction was estimated from the intensity of the $g = 2.09$ EPR signal from nitrosylferrocytochrome a_3.

These observations are consistent with the slow reduction of the oxidized enzyme by NO. However, we observed the reduction of only cytochrome a_3 and Cu_{a_3}; cytochrome a and Cu_a remained fully oxidized even after long incubation times. Moreover, it appears that the reduction proceeds via a two-electron step, since no one-electron reduced intermediates (e.g., nitrosylferrocytochrome $a_3/Cu_{a_3}^{2+}$) were observed.

The reduction of the oxidized enzyme by NO was confirmed by following the optical spectrum. For incubation times <30 min, NO had no effect on the optical spectrum of the oxidized enzyme (Figure 11). Thereafter, the optical spectrum slowly changed with time. The spectrum obtained after several hours of incubation at room temperature is shown in Figure 11. This spectrum, which has a Soret band at 426 nm and an α band at 597 nm, is nearly identical with that which was observed when the reduced enzyme was reoxidized by NO (cf. Figure 4). The intensity of the α band in this spectrum was intermediate between that in the fully oxidized enzyme and the one-quarter reduced enzyme (Stevens et al., 1979a). In the light of the EPR results presented earlier, these optical results are best interpreted in terms of a steady state containing a mixture of the fully oxidized enzyme and the two-electron reduced NO-bound enzyme.

It is interesting to compare the reduction of cytochrome *c* oxidase by NO in the presence and absence of azide. In the former case, it was shown that the enzyme was reduced via two consecutive one-electron steps (reactions 5 and 6). However, in the latter case, the enzyme was reduced either via a two-electron step or via two one-electron steps which occur in rapid succession, since no one-electron reduced states

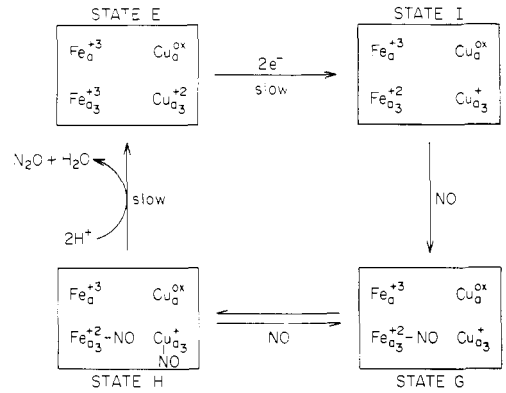

FIGURE 12: The proposed reaction cycle which occurs when oxidized cytochrome *c* oxidase is incubated with NO.

were observed. Earlier in this work we showed that the enzyme can catalyze the oxidation of NO to nitrite (reaction 6). However, this one-electron step was quite slow, and it would not be expected to occur twice in rapid succession. Therefore, the reduction of the oxidized enzyme must have occurred via a two-electron process. One possibility is reaction 8. Un-

$$NO + H_2O \rightarrow NO_2 + 2H^+ + 2e^-(\text{enzyme}) \qquad (8)$$

fortunately, due to the slow rate of reduction of the oxidized enzyme by NO, we have been unable to detect any products by mass or NMR spectroscopy which would allow us to verify this redox process. However, we were able to observe the production of N_2O by mass spectroscopy which indicates that the enzyme was reoxidized via reaction 4.

Alternatively, the enzyme could be reduced by endogenous reductants which may be present as impurities in preparations of the enzyme (Powers et al., 1979). To investigate this possibility, we examined the EPR spectrum as a function of time of a sample of the anaerobic oxidized enzyme which was incubated at 4 °C. Even after 4 days of incubation, we found no indication that autoreduction had occurred. In particular, we observed no reduction in the cytochrome *a* or Cu_a EPR signal intensities or increase in high-spin heme EPR signals from cytochrome a_3 which are known to appear when the enzyme is partially reduced (Hartzell & Beinert, 1976). Although this experiment would seem to rule out the possibility of reduction by endogenous reductants, this point cannot be totally settled until the nature and presence of such endogenous reductants (Powers et al., 1979) are better defined.

Regardless of the source of electrons which reduce cytochrome *c* oxidase, it is clear that the enzyme undergoes a cycle of oxidation and reduction in the presence of NO. Such a cycle is depicted in Figure 12. This cycle is analogous to that which occurred when azide and NO were added to the oxidized enzyme except that here the reduction of the enzyme occurs via reaction 8 or possibly, although less likely, via endogenous reductants. Both our EPR and optical results indicated that the steady state, in the presence of excess NO, contained a mixture of states E and H. Therefore, in this case both the reduction and the oxidation of the enzyme by NO must have been comparably slow.

Discussion

We have found that cytochrome *c* oxidase catalyzes (1) the reduction of two NO molecules to N_2O (reaction 4), (2) the reaction of NO plus azide to form N_2O and N_2 (reaction 5), (3) the reversible oxidation of NO to nitrite (reactions 6 and 7), and probably also (4) the oxidation of NO to NO_2 (reaction 8).

Both reactions 4 and 7 have been observed to be catalyzed by other metalloproteins. Hemocyanin (Verplaetse et al., 1979) and cytochrome *cd* (Matsubara & Iwasaki, 1972) in particular are known to catalyze the reduction of two NO molecules to N_2O. In addition, under anaerobic conditions a number of bacterial organisms are capable of utilizing NO as the terminal electron acceptor. In these bacteria it is believed that the reductive pathway consists of a sequential reduction of nitrate → nitrite → NO → N_2O → N_2 (St. John & Hollocher, 1977). Although the enzymes involved in this denitrification process have not been well characterized, it appears that many are cytochromes (Cox et al., 1971). Hence, although the primary function of cytochrome *c* oxidase is to reduce oxygen to water, it is not surprising that this enzyme also possesses some rudimentary nitric oxide reductase and nitrite reductase activity.

Nonetheless, these reactions provide new information on the catalytic activity of cytochrome *c* oxidase. In all of the reactions of NO observed in this work, a stable product was formed after a one- or two-electron step. These reactions can be contrasted to the reduction of dioxygen to water by the enzyme, which involves four electrons. In the latter process, partially reduced species are not released from the enzyme. These one- and two-electron reactions of NO, therefore, may shed some light on the initial one- or two-electron reduction of dioxygen. In particular, the states of cytochrome *c* oxidase which are formed in the course of reaction with NO and monitored by EPR may be indicative of the states formed during the reduction of dioxygen to water.

The reactions of NO with the enzyme occur exclusively at the cytochrome a_3/Cu_{a_3} site with no apparent involvement of either cytochrome *a* or Cu_a. For example, in the reaction of the oxidized enzyme with azide and NO, the reduction and reoxidation of cytochrome a_3 and Cu_{a_3} occurs with no detectable effect on the EPR signals of either cytochrome *a* or Cu_a. This result supports the view that the cytochrome a_3/Cu_{a_3} site is not intimately interacting with the other two metal centers during the catalytic process.

Our NO experiments have also provided some definite information on the structure of the oxygen binding site. In the oxidized enzyme, NO readily binds to Cu_{a_3}, even while fluoride or cyanide remain bound to cytochrome a_3 (Stevens et al., 1979a). Thus, the ligand binding pocket in the oxidized enzyme must be large enough to accommodate both NO and cyanide. It is possible that cyanide or fluoride bridges between cytochrome a_3 and Cu_{a_3} in these complexes with NO occupying a second binding site on Cu_{a_3} as suggested by Chan et al. (1980).

Of greater significance to the catalytic function of the enzyme is the structure of the oxygen binding site in the reduced protein. Our results indicate that two NO molecules can bind to the reduced enzyme; one is tightly bound to cytochrome a_3 and the other is weakly bound to Cu_{a_3}. It is clear that in the di-NO complex the two bound NO molecules interact strongly, since this state is EPR silent. Thus, the binding sites on cytochrome a_3 and Cu_{a_3} must be quite close to each other.

It is possible to estimate the distance between cytochrome a_3 and Cu_{a_3} from the magnitude of *D*, the zero-field splitting, in the nitrosylferrocytochrome $a_3/Cu_{a_3}^{2+}$ triplet state. We obtained a value for $|D|$ of ~ 0.07 cm^{-1} from the breadth of the $\Delta Ms \pm 1$ transition for this triplet (Stevens et al., 1979a). Assuming a purely dipolar interaction between the two spins, we calculate a distance of 3.4 Å between the two spin centers (Symons, 1978). However, spin–orbit and exchange interactions can also contribute to the zero-field splitting in triplets.

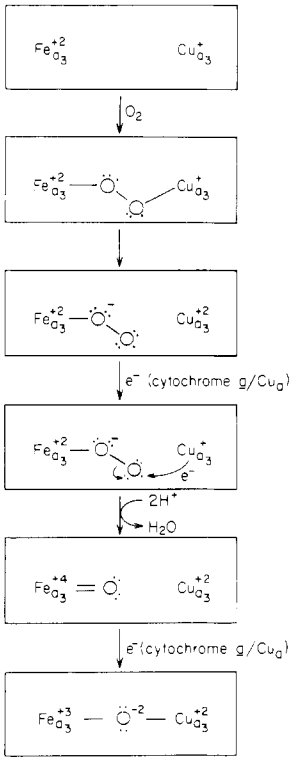

FIGURE 13: Proposed mechanism for the reduction of dioxygen to water by cytochrome *c* oxidase.

These contributions are of the order $(\Delta g/g)J_i$ and $(\Delta g/g)^2J_i$, respectively (Abragam & Bleaney, 1970), where J_i is the isotropic superexchange energy. By use of the average of the g values for nitrosylferrocytochrome a_3 (Blokzijl-Homan & Van Gelder, 1971) and Cu_{a_3} (Malmström et al., 1980), $\Delta g/g$ can be estimated to be 0.04. There is, however, a major uncertainty in the magnitude of J_i. The temperature dependence of the triplet signal shows no deviation from the expected Boltzmann distribution of the spins among the triplet sublevels from 7 to 80 K (S. I. Chan, unpublished results). This result may be taken to infer that $|J| \leq 5$ cm^{-1}, where J is the singlet–triplet splitting. However, the zero-field splitting of the triplet depends on the exchange interaction, J_i, between the ground state of one site and an excited state, which is coupled to the ground state through spin–orbit coupling, of the second site (Abragam & Bleaney, 1970). The magnitude of J_i is expected to be much less than J, particularly for a triplet involving a nitrosylferroheme species. Accordingly, the spin–orbit and exchange contributions to the zero-field splitting in the nitrosylferrocytochrome $a_3/Cu_{a_3}^{2+}$ triplet state are likely to be quite small. Taking these considerations into account and the fact that the spin on nitrosylferrocytochrome a_3 lies substantially on the nitrogen of NO, we estimate that the distance between cytochrome a_3 and Cu_{a_3} is ~5 Å, which is close to that expected if dioxygen bridges between the two metals.

The close proximity of the two metals may be important in the stabilization and anchoring of reactive intermediates which are formed during the reduction of dioxygen to water. This close proximity is undoubtedly also important in the catalysis of the reactions with NO discussed in this work. In particular, reactions 4 and 5 must be catalyzed by the binding of two molecules in close proximity and by maintaining this close association at the oxygen binding site for a sufficiently long time to allow for the redox reactions to occur.

Finally, we consider the implications of our NO experiments on the mechanism of oxygen reduction. In this regard, it is important to compare the reduction of the protein under aerobic and anaerobic conditions. When cytochrome *c* oxidase is reduced anaerobically, Cu_{a_3} is reduced before cytochrome a_3. Also, when the fully reduced enzyme is reoxidized anaerobically, cytochrome a_3 is oxidized before Cu_{a_3}. These differences in reduction potentials between the two metal centers have allowed cytochrome a_3 to be observed by EPR as a high-spin heme in a state where cytochrome a_3 is oxidized while Cu_{a_3} is reduced (Shaw et al., 1978). However, it is clear from this work that in the presence of NO, the reduction potential of cytochrome a_3 becomes higher than that of Cu_{a_3}. This raises the question of whether or not the reduction potentials measured on the anaerobic enzyme have any direct bearing on the enzyme during its reaction with dioxygen. Presumably the coordination of dioxygen to the enzyme would also greatly perturb the reduction potential of cytochrome a_3. In fact, it is possible that the nitrosylferrocytochrome $a_3/Cu_{a_3}^{2+}$ triplet state, which is stabilized by NO, resembles a state formed during the reaction of the enzyme with dioxygen. Thus the role of cytochrome a_3 may be to anchor dioxygen to the enzyme while remaining in the ferrous state, and the role of Cu_{a_3} may be to receive electrons from cytochrome a/Cu_a and sequentially transfer them to dioxygen.

In the light of these considerations, we propose the mechanism for oxygen reduction shown in Figure 13. The reduction of oxygen is proposed to proceed in five steps. (1) Dioxygen binds to ferrocytochrome a_3 and forms a bridge with Cu_{a_3}. (2) In this state the electron on Cu_{a_3} is rapidly delocalized onto the oxygen. (3) Cu_{a_3} then accepts an electron from cytochrome a or Cu_a. (4) This reduction of Cu_{a_3} results in the immediate splitting out of one water molecule and the formation of a ferrylcytochrome a_3–O^{2-} state. This state involving an Fe(IV) is proposed to be only a transient intermediate formed during the reduction of dioxygen and, as such, has been proposed before in a number of systems including cytochrome *c* oxidase (Chance et al., 1975; Collman et al., 1977). (5) Finally, another electron from cytochrome a or Cu_a is transferred to cytochrome a_3 to fully oxidize the enzyme and form a μ-oxo bridge between cytochrome a_3 and Cu_{a_3}. As was suggested by Blumberg & Peisach (1979), this μ-oxo bridge between cytochrome a_3 and Cu_{a_3} could facilitate the strong antiferromagnetic exchange interaction between these two metal centers in the oxidized enzyme. Our proposed mechanism differs from previously proposed mechanisms in that a μ-peroxo intermediate state is not formed during the reduction of dioxygen to water. It may be that the oxygen binding site in cytochrome *c* oxidase involves two nonequivalent metal centers to avert the formation of a μ-peroxo species.

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Essential Sulfhydryl for Reduced Nicotinamide Adenine Dinucleotide Binding in D-β-Hydroxybutyrate Dehydrogenase†

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ABSTRACT: Chemical derivatization studies have been directed at the sulfhydryl group of D-β-hydroxybutyrate dehydrogenase, a lipid-requiring enzyme. Reaction with *N*-ethylmaleimide leads to progressive and parallel loss of both enzymic activity and coenzyme binding. Both functions are lost when 1 equiv of sulfhydryl is derivatized per mol of enzyme. Inactivation of the enzyme with methylmercury or with air oxidation also leads to loss of coenzyme binding. We conclude that a single "essential" sulfhydryl is required for coenzyme binding and consequently for enzymic activity. Only two "accessible" cysteine residues can be derivatized even at high levels of *N*-ethylmaleimide, whereas derivatization of the remaining three "inacessible" cysteines requires denaturation of the enzyme. The enzyme can apparently be labeled in the accessible, but nonessential, sulfhydryl in the presence of coenzyme which protects against inactivation by *N*-ethylmaleimide. Such selective covalent labeling of the nonessential sulfhydryl makes possible future biophysical studies of enzyme–phospholipid interaction of a functional enzyme using extrinsic probes.

D-β-Hydroxybutyrate dehydrogenase is a lipid-requiring enzyme with an absolute requirement of lecithin for function. The apodehydrogenase, which is devoid of lipid, has been purified to homogeneity. It is inactive, but can be made functional by forming an active enzyme–phospholipid complex. For a review, see Fleischer et al. (1974).

D-β-Hydroxybutyrate dehydrogenase is perhaps the most extensively studied lipid-requiring enzyme, and we are studying it to understand the molecular basis of the role of lipid (Gazzotti et al., 1974) and the nature of lipid–protein interaction (Fleischer et al., 1979). In this regard, the chemistry of this enzyme is now being addressed, including primary sequence and chemical derivatization studies in collaboration with Dr. Kenneth Mann of the Mayo Foundation. In the present study, we demonstrate the importance of a single sulfhydryl moiety for both enzymic function and coenzyme (NADH)[1] binding. A preliminary report of this work has appeared (Latruffe et al., 1979).

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[1] Abbreviations used: BAL, British Anti-Lewisite (2,3-mercapto-1-propanol); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MalNEt, *N*-ethylmaleimide; Mops, 4-morpholinepropanesulfonic acid; MPL, mitochondrial phospholipids; NAD(H), nicotinamide adenine dinucleotide (reduced); Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EPR, electron paramagnetic resonance.

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