of numerous membrane components. These findings suggest that if the 170K dalton protein is the receptor for EGF, the concentrations of membrane receptors remaining after down regulation are sufficient to produce near maximal phosphorylation of numerous membrane components. Thus, additional studies are required to elucidate this aspect of the mechanism of action of EGF alteration of hormonal responsiveness in vitro.

The results presented here, taken together with published reports (Fox & Das, 1979; Fox et al., 1979; Carpenter & Cohen, 1979; Greengard, 1978; Heldin et al., 1979), suggest that modulation of receptor number and membrane phosphorylation may be a general mechanism for regulation of cellular responsiveness to hormones. Whether down regulation and reduction of phosphorylation of a protein of M_r 170K represent different aspects of the same phenomenon remains to be determined. It seems possible that the specific apparent reduction of phosphorylation of the 170K M_r phosphoprotein mediated by EGF may regulate the hormonal sensitivity of NRK cells to EGF. Finally, it is conceivable that the 170K-dalton phosphoprotein is a component of the EGF receptor of NRK cells which is a substrate of the phosphorylation reaction.

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Conformations of Oxidized Cytochrome *c* Oxidase†

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ABSTRACT: Oxidized cytochrome *c* oxidase is shown to exist in three conformations in addition to the transient "*g*5" conformation previously reported [Shaw, R. W., Hansen, R. E., & Beinert, H. (1978) *J. Biol. Chem. 253*, 6637–6640]. The "resting" and "*g*12" conformations are distinguished by an NO-induced cytochrome a_3 electron paramagnetic resonance (EPR) signal and an EPR signal at $g' = 12$, respectively. The "oxygenated" conformation exhibits an unusual EPR signal in the presence of fluoride and is identical with the "oxygenated" state first discovered by Okunuki et al. [Okunuki, K., Hagihora, B., Sekuzu, I., & Horio, T. (1958) *Proc. Int. Symp. Enzyme Chem., Tokyo, Kyoto*, 264]. It is proposed that when the reduced enzyme is reoxidized by dioxygen, the *oxidized* enzyme first relaxes from the "*g*5" into the "oxygenated" conformation after which a percentage of the molecules slowly relax into the "*g*12" conformation. The "resting" conformation is not formed when the enzyme is reoxidized. On the basis of the EPR observations, it is proposed that these various conformations of the oxidized enzyme differ in the structure of the cytochrome a_3–Cu_{a_3} site. Structures for the cytochrome a_3–Cu_{a_3} site are proposed for each conformation, and a mechanism by which these conformations undergo interconversion among themselves is described.

Oxidized cytochrome *c* oxidase can exist in more than one conformation (Muijsers et al., 1971; Antonini et al., 1977; Rosén et al., 1977; Shaw et al., 1978; Petersen & Cox, 1980). Two classes of conformations have been recognized: those that are present in the oxidized enzyme as isolated and those that are transiently formed when the reduced enzyme is reoxidized with O_2. At this time these two classes of conformations have been collectively referred to as the "resting" and "oxygenated" (also "pulsed") conformations, respectively. The distinction between the two classes is that the resting conformation reacts slowly with O_2 when mixed with both reductant and O_2, whereas the pulsed enzyme reacts rapidly (Antonini et al., 1977; Rosén et al., 1977). However, the question of the

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structural differences between these conformations and even the number of distinct species comprising each class of conformations has not been resolved.

The position of the Soret absorption maximum has been the criterion by which the resting and oxygenated classes of conformations were originally distinguished (Okunuki et al., 1958). The oxidized enzyme as isolated exhibits a Soret maximum at 418–424 nm (Lemberg, 1969). The range of values for the Soret maximum of the resting class of the oxidized enzyme among various preparations of the enzyme is a clear indication that more than one distinct conformation is present in the enzyme as isolated and the proportions of each conformation vary among preparations of the enzyme. In contrast, the oxygenated class of the oxidized enzyme exhibits a Soret maximum at 428 nm (Muijsers et al., 1971; Shaw et al., 1978). The oxygenated class of the oxidized enzyme, however, also contains at least two distinct conformations that appear to form sequentially when the reduced enzyme is reoxidized by O_2. Within 5 ms after adding O_2 to the reduced enzyme, a state is formed that has a Soret maximum at 428 nm and exhibits a "novel EPR signal" with resonances at $g = 5$, 1.78, and 1.69 (Shaw et al., 1978). This state decays with a half-time of 100 s at 16 °C into a state which no longer exhibits the $g = 5$, 1.78, and 1.69 EPR[1] signal but which still exhibits a Soret maximum at 428 nm. The conformation formed about 100 s after reoxidation with O_2 can be identified as the oxygenated state first discovered by Okunuki et al. (1958). While the original notion of the oxygenated state being an enzyme–oxygen complex is no longer tenable, the designation oxygenated has persisted, and we shall continue to refer to the state formed in about 100 s after reoxidation with O_2 in this manner. It is generally assumed that the oxygenated state itself decays into one or more of the resting conformations with a half-time on the order of 1 h at pH 7.4 and 21 °C (Lemberg & Stanbury, 1967). However, the resting enzyme obtained after a cycle of reduction and reoxidation by O_2 often does not exhibit a Soret maximum at the same wavelength as did the same sample before being reduced (Muijsers et al., 1971).

In this paper, we have utilized NO as a spin probe to study the conformations of oxidized cytochrome *c* oxidase by EPR. Previous work from this laboratory has shown that NO induced a high-spin cytochrome a_3 EPR signal from the oxidized enzyme but that the intensity of this signal varied among preparations of the enzyme, accounting for as much as 60% of one heme to less than 5% of one heme (Stevens et al., 1979). It was surmised that this variation in the intensity of the NO-induced cytochrome a_3 EPR signal arose from a conformational heterogeneity of the oxidized enzyme as isolated. We have now investigated the nature of this conformational heterogeneity and have found that the oxidized enzyme as isolated contains variable proportions of three distinct conformations. Methods are described that allow each conformation to be distinguished and quantitated. The ligand binding and EPR properties of these conformations indicate that the differences between the conformations can be understood in terms of differences in the structure of the O_2 binding site. The mechanism by which these conformations undergo interconversion among themselves and the functional significance of each are discussed.

[1] Abbreviations used: EPR, electron paramagnetic resonance; PPD, *p*-phenylenediamine.

Materials and Methods

Sample Preparation. Beef heart cytochrome *c* oxidase was isolated by the procedures of Hartzell & Beinert (1974) and Yu et al. (1975). The purified protein was dissolved in 50 mM Tris/acetate buffer, pH 7.4, and 0.5% Tween 20 (Hartzell and Beinert preparation) and in 50 mM phosphate, pH 7.4, and 0.5% cholate (Yu et al. preparation). Both preparations yielded samples of the enzyme with 9–11 nmol of heme *a*/mg of protein as measured by the pyridine hemochromogen assay (Takemori & King, 1965; Lowry et al., 1951). The concentrations of the enzyme quoted in this work were based on 2 heme *a*/enzyme.

Cytochrome *c* oxidase activities were measured at 30 °C with a YSI oxygen electrode in the presence of 0.5% Tween 80 (Vik & Capaldi, 1980). The isolated enzyme preparations used in this study showed activities, in the presence of detergent, ranging from 90 to 120 mol of cytochrome *c* (mol of cytochrome *c* oxidase)$^{-1}$ s^{-1}.

The addition of NO to the samples is described in a recent paper (Brudvig et al., 1980). In addition to the oxidized enzyme plus NO complex, five other complexes of cytochrome *c* oxidase were prepared in this work: (i) oxidized enzyme plus cyanide, (ii) oxidized enzyme plus cyanide and NO, (iii) oxidized enzyme plus fluoride, (iv) oxidized enzyme plus fluoride and NO, and (v) the "oxygenated" enzyme.

The oxidized enzyme plus cyanide was prepared by the addition of a 1:2 mol ratio mixture of solid KCN and KH_2PO_4 to an anaerobic sample from a side arm on the EPR tube. This mixture resulted in a solution with pH 7.4 when added to the enzyme. The amount of KCN added would have given a final KCN concentration of 100 mM. However, the major portion of the cyanide bubbled off as HCN. The actual concentration of dissolved cyanide, estimated from the partial pressure of HCN over the sample and the solubility of HCN in water, was about 2 mM. The oxidized enzyme plus cyanide and NO complex was prepared as above except that the KCN plus KH_2PO_4 mixture was added to the enzyme while the sample was under vacuum. Then NO was admitted to the anaerobic sample after the desired period of incubation to give a final pressure of 1 atm without further evacuation of the enzyme solution. It was found that most of the cyanide in the sample was removed if the enzyme was degassed after the addition of KCN, resulting in incomplete formation of the oxidized cyanide complex.

The oxidized enzyme plus fluoride was prepared by adding an aliquot of a 1 M solution of KF to the oxidized enzyme to give the desired final fluoride concentration and then making the sample anaerobic. The oxidized enzyme plus fluoride and NO complex was prepared in the same manner as the oxidized enzyme plus fluoride with the further addition of NO to a pressure of 1 atm.

The oxygenated state of the enzyme was prepared by adding a 5- to 10-fold excess of ascorbate plus PPD to reduce the anaerobic enzyme and then reoxidizing the enzyme with air. In the experiments in which the rate of decay of the oxygenated enzyme was monitored by EPR, individual samples were reduced with ascorbate plus PPD and then placed in a dialysis bag and dialyzed against 1000 volumes of an aerobic buffer solution to reoxidize the enzyme and to remove the ascorbate and PPD from the sample. The beginning of the dialysis was taken as the zero of time and samples were removed at various times, immediately placed in an EPR tube, and frozen in liquid nitrogen.

EPR. The EPR spectra were recorded on a Varian E line century series X-band EPR spectrometer equipped with an

Air Products Heli-Trans low-temperature system. The intensity of the high-spin ferricytochrome a_3 EPR signals was determined relative to an external metmyoglobin standard (dissolved in 10 mM phosphate, pH 6.0) and also relative to the low-spin ferricytochrome *a* EPR signal. The high-spin heme EPR signals were integrated by the method of Aasa et al. (1976). The low-spin cytochrome *a* EPR signal was integrated by the method of Aasa & Vänngård (1975), using the $g = 3.0$ component to determine the total area. The low-spin cytochrome *a* EPR signal has been shown to correspond to 100% of one heme (Aasa et al., 1976), and on this basis the low-spin cytochrome *a* EPR signal was used as an internal standard. The intensity of the *rhombic* high-spin cytochrome a_3 EPR signal induced by NO, as determined by using a metmyoglobin standard, was found to be independent of temperature. This result indicates that the zero-field splitting parameter (D) is nearly equal for these two high-spin ferric hemes. D has been measured to be 9.1 cm^{-1} for metmyoglobin–H_2O (Scholes et al., 1971). Accordingly, we have taken D to be 9 cm^{-1} for the rhombic high-spin cytochrome a_3 EPR signal induced by NO. However, for metmyoglobin–fluoride, D was found to be 6.1 cm^{-1}, substantially smaller (Scholes et al., 1971). We therefore have assumed that $D = 6$ cm^{-1} for the more axial high-spin cytochrome a_3 EPR signals induced by NO in the presence of fluoride. After correction for the distribution of population among the spin sublevels of the high-spin ferric heme, the high-spin cytochrome a_3 EPR intensities determined by using the internal cytochrome *a* standard were found to agree with those determined by using the metmyoglobin standard to within 10%.

The intensity of the low-spin cyanoferricytochrome a_3 EPR signal was determined relative to the low-spin cytochrome *a* EPR signal. All three *g* values of the cyanocytochrome a_3 EPR signal are not known since the signal is very anisotropic and the two high-field turning points have not been observed. Therefore, the cyanocytochrome a_3 EPR signal was integrated by the method of DeVries & Albracht (1979) using the $g = 3.5$ component to determine the total area.

Under appropriate conditions, cytochrome *c* oxidase exhibits a broad EPR signal[2] at $g' = 12$ (Greenaway et al., 1977; Beinert & Shaw, 1977). This EPR signal is quite unusual for heme or copper proteins; in fact, at the present time it is not known for sure that it is associated with any one of the metal centers in cytochrome *c* oxidase, although its intensity has been observed to increase as the purification of the enzyme proceeds (Greenaway et al., 1977). Also, it has not been possible to quantitate the $g' = 12$ EPR signal directly because it is not known with certainty whether other resonances at higher or lower fields are associated with the $g' = 12$ EPR signal (in this regard, the $g' = 12$ EPR signal does not appear to have equal area above and below the base line in its first derivative spectrum). We have obtained an empirical formula for calculating the fraction, F, of enzyme molecules exhibiting the EPR signal at $g' = 12$ from the data presented in this paper (eq 1). In this formula, $H_{g'=12}$ (16 K) is the maximum-to-minimum height in millimeters of the $g' = 12$ EPR signal at 16 K, $A_{g=3}$ (16 K) is the area of the $g = 3.0$ component of the cytochrome *a* EPR signal at 16 K calculated by summing the peak heights in millimeters at 10-G spacings, and 17 is the proportionality constant. The cytochrome *a* EPR signal was used as an internal standard to obtain this formula, since its intensity remained constant throughout the experiments described in this work and represented one heme/enzyme. Equation 1 is only appropriate when the EPR signals are recorded at 16 K. If the EPR signals are recorded at a higher or lower temperature, T, it is necessary to scale the observed $H_{g'=12}(T)$ and $A_{g=3}(T)$ to 16 K; $A_{g=3}(T)$ can be scaled to 16 K by multiplying $A_{g=3}(T)$ by the Boltzmann factor. However, $H_{g'=12}(T)$ was found to be significantly more temperature dependent (Figure 1). This temperature dependence most likely arises from the $g' = 12$ EPR signal being associated with an $S > {}^1/_2$ species, but it may also be due in part to broadening of the signal observed at higher temperatures. Accordingly, $H_{g'=12}(T)$ was scaled to 16 K by using the data summarized in Figure 1.

$$F = 17\frac{H_{g'=12}\ (16\ \text{K})}{A_{g=3}\ (16\ \text{K})} \qquad (1)$$

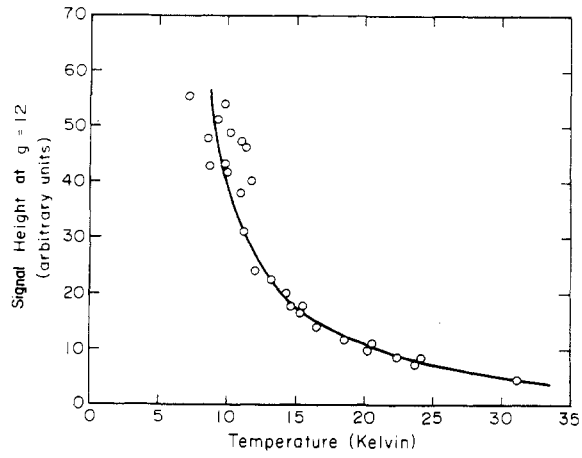

FIGURE 1: The maximum-to-minimum height of the EPR signal centered at $g' = 12$ vs. the temperature. Conditions: microwave power, 0.2 mW; modulation amplitude, 16 G; microwave frequency, 9.24 GHz.

Optical Spectroscopy. Optical measurements were carried out at room temperature on a Beckman Acta CIII spectrometer.

Results

In this work we will focus on the differences in the interaction of NO with two types of preparations of the enzyme: the Hartzell and Beinert preparation and Yu et al. preparation. By careful analysis of the EPR signals exhibited by these two preparations, we will demonstrate the existence of at least three conformations of oxidized cytochrome *c* oxidase as isolated from beef heart. A fourth conformation of the oxidized enzyme that is rapidly and transiently formed when the reduced enzyme is reoxidized by O_2 has been identified by Shaw et al. (1978). The EPR properties of these four conformations of oxidized cytochrome *c* oxidase are listed in Table I. We have named the four conformations as follows: (i) the "*g*12" conformation (which exhibits a $g' = 12$ EPR signal); (ii) the "*g*5" conformation (which exhibits an EPR signal with resonances at $g = 5$, 1.8, and 1.7); (iii) the oxygenated conformation (in which fluoride *alone* induces a fluorocytochrome a_3–Cu_{a_3} EPR signal); and (iv) the resting conformation (in which NO *alone* induces a high-spin cytochrome a_3 EPR signal). The appropriateness of these names will become apparent as the results are presented.

Oxidized Enzyme Plus NO. The two preparations investigated differed in that NO induced a high-spin cytochrome a_3 EPR signal [see Figure 1 in Stevens et al. (1979)] ac-

[2] The *g* value for this resonance is only an effective *g* value, since its value depends on the microwave frequency used to observe the EPR signal (W. E. Blumberg, personal communication). Hence, we will label the *g* value with a prime. This point will be discussed in a later section.

Table 1: EPR Properties of the Conformations of Oxidized Cytochrome *c* Oxidase[a]

state	conformation			
	"*g*12"	resting	oxygenated	"*g*5"[b]
unligated	$g' = 12$ EPR signal	EPR silent	EPR silent	$g = 5, 1.8, 1.7$ EPR signal
plus NO	$g' = 12$ EPR signal	high-spin cyt a_3^{3+} EPR signal	EPR silent	*c*
plus fluoride	$g' = 12$ EPR signal	EPR silent	cyt a_3^{3+}–F^-–$Cu_{a_3}^{2+}$ EPR signal	*c*
plus fluoride and NO	$g' = 12$ EPR signal	high-spin cyt a_3^{3+} EPR signal	high-spin cyt a_3^{3+}–F^- EPR signal	*c*
plus cyanide	$g' = 12$ EPR signal *slowly* eliminated	EPR silent	EPR silent	$g = 5, 1.8, 1.7$ EPR signal eliminated
plus cyanide and NO	low-spin cyt a_3^{3+}–CN^- EPR signal *slowly* appears	low-spin cyt a_3^{3+}–CN^- EPR signal	low-spin cyt a_3^{3+}–CN^- EPR signal	*c*

[a] The distinguishing property of each conformation is underlined. [b] Shaw et al. (1978). [c] Not determined.

counting for 60% of one heme in the Hartzell and Beinert preparation but only 1% of one heme in the Yu et al. preparation. The fraction of cytochrome a_3 observed by EPR in the presence of NO was unchanged after a variety of treatments. For example, changing the pH from 7.0 to 9.0 did not change the intensity of the cytochrome a_3 EPR signal induced by NO. Other treatments, such as changing the ionic strength of the buffer, changing the detergent in which the enzyme was dissolved from Tween 20 to cholate, or precipitating the enzyme by ammonium sulfate and resolubilizing the enzyme in phosphate buffer, also did not alter the intensity of the cytochrome a_3 EPR signal induced by NO.

Effect of NO on the $g' = 12$ EPR Signal. When NO was added to the oxidized enzyme, the intensity of the $g' = 12$ EPR signal did not change, even when a large high-spin cytochrome a_3 EPR signal was induced. Moreover, the intensity of the $g' = 12$ EPR signal was about 2-fold larger in the Yu et al. preparation than in the Hartzell and Beinert preparation. This observation can be contrasted with the intensity of the NO-induced cytochrome a_3 EPR signal which was much larger in the Hartzell and Beinert preparation than in the Yu et al. preparation. It thus appears that the $g' = 12$ EPR signal must arise from molecules distinct from those in which NO induced a high-spin cytochrome a_3 EPR signal.

Oxidized Enzyme Plus Cyanide and NO. Stevens et al. (1979) found that cyanide plus NO induced a low-spin cyanocytochrome a_3 EPR signal from oxidized cytochrome *c* oxidase. It was concluded (Chan et al., 1980) that NO was bound to Cu_{a_3} while cyanide was bound to cytochrome a_3; NO acted to uncouple Cu_{a_3} from cytochrome a_3 and cyanide induced a high- to low-spin transition when bound to cytochrome a_3. The intensity of the low-spin cyanocytochrome a_3 EPR signal induced by NO and cyanide was equal to that of the high-spin cytochrome a_3 EPR signal induced by NO alone. Thus cyanide appears to bind quantitatively to cytochrome a_3 in the enzyme molecules in which NO induces a cytochrome a_3 EPR signal.

We found that the addition of cyanide to the oxidized Yu et al. preparation of the enzyme had no effect on the $g' = 12$ EPR signal provided that the sample was mixed and *immediately* frozen. However, when the oxidized enzyme was incubated at 4 °C in the presence of cyanide, the $g' = 12$ EPR signal gradually disappeared (Figure 2). This result demonstrates that the $g' = 12$ EPR signal is associated with the cytochrome a_3–Cu_{a_3} site and that cytochrome a_3 does not readily bind exogenous ligands in those molecules that exhibit the $g' = 12$ EPR signal.

It was possible to directly monitor the fraction of cyanide-bound enzyme molecules in the above experiment by adding NO to our sample of the Yu et al. preparation.[3] A

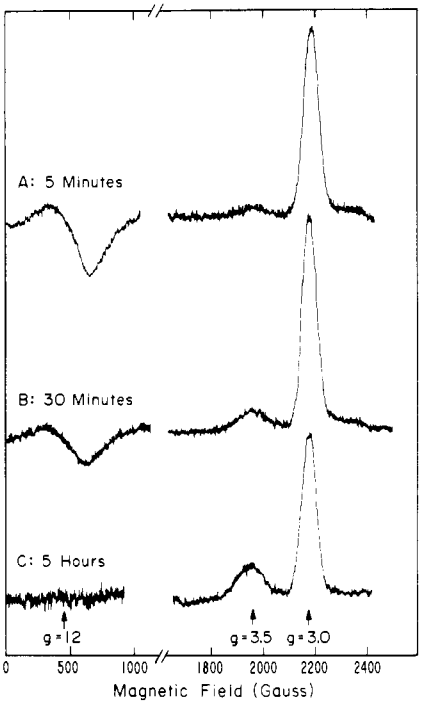

FIGURE 2: EPR spectra of the Yu et al. (1975) preparation of cytochrome *c* oxidase in the presence of both cyanide and NO. In all cases 1 atm of NO was added and mixed with the sample for 2 min, and then the sample was immediately frozen at 77 K. The samples contained 0.2 mM cytochrome *c* oxidase and were preincubated with approximately 2 mM HCN at 4 °C before adding NO: (A) 5-min preincubation; (B) 30-min preincubation; and (C) 5-h preincubation. The low-field ($g = 12$) portions of the signals were recorded at (A) 3.2×10^4 gain, 14 K; (B) 3.2×10^4 gain, 16.5 K; (C) 8×10^4 gain, 17.5 K. The high-field portions were recorded at (A) 2.5×10^4 gain, 16 K; (B) 3.2×10^4 gain, 16.5 K; (C) 3.2×10^4 gain, 17.5 K. All spectra were recorded at 0.5-mW power, 16-G modulation amplitude, and 9.23-GHz frequency.

direct parallel was found between the *decrease* in the intensity of the $g' = 12$ EPR signal and the *increase* in the intensity of the NO-induced cyanocytochrome a_3 EPR signal when the oxidized enzyme was incubated with cyanide (Figures 2 and 3). After long incubation times (more than 6 h), the $g' = 12$ EPR signal was completely eliminated and the intensity of the NO-induced cyanocytochrome a_3 EPR signal accounted

[3] In order for intensity of the NO-induced cyanocytochrome a_3 EPR signal to be a true indication of the fraction of enzyme molecules with bound cyanide, the sample must be mixed with NO and immediately frozen. When the sample was incubated at 4 °C in the presence of NO, the intensity of the NO-induced cyanocytochrome a_3 EPR signal decreased. This observation can be explained if NO induced a slow reduction of the enzyme [see Brudvig et al. (1980)].

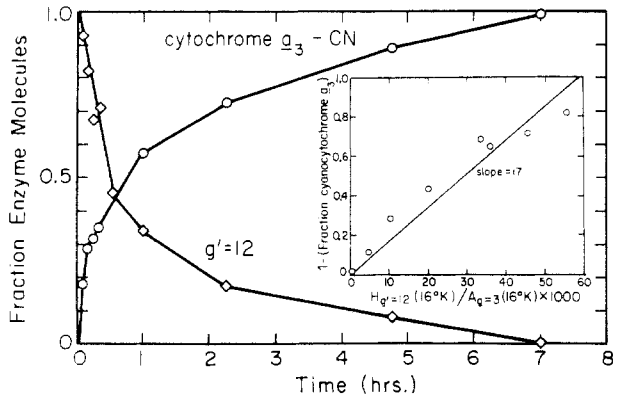

FIGURE 3: Fraction of the Yu et al. (1975) preparation of cytochrome *c* oxidase which exhibited an EPR signal at $g' = 12$ (determined from eq 1) and the fraction in which NO induced a low-spin cyanocytochrome a_3 EPR signal at $g = 3.5$ as a function of time of preincubation with cyanide. The concentrations and EPR conditions were the same as in Figure 2A. Insert: Fraction of the enzyme molecules which did *not* show a cyanocytochrome a_3 EPR signal vs. the ratio of the maximum-to-minimum height in mm of the $g' = 12$ EPR signal at 16 K, $H_{g'=12}$ (16 K), to the area of the $g = 3.0$ component of the cytochrome *a* EPR signal at 16 K, $A_{g=3}$ (16 K). The values measured for $H_{g'=12}$ were normalized to 16 K by using the data in Figure 1; the areas of the $g = 3.0$ peak were calculated by summing the peak heights in mm at 10-G intervals and were normalized to 16 K by multiplying by the Boltzmann factor. The measured slope of 17 was then used in eq 1 to calculate the fraction of enzyme molecules exhibiting an EPR signal at $g' = 12$.

for 100% of one heme. It should be emphasized that the cyanocytochrome a_3 EPR signal only appeared when NO was added to the sample in the presence of cyanide and that the cyanocytochrome a_3 EPR signal was completely eliminated upon removal of NO from the sample. The removal of NO did not restore the $g' = 12$ EPR signal.

The direct parallel between the decrease in intensity of the $g' = 12$ EPR signal and the increase in intensity of the NO-induced cyanocytochrome a_3 EPR signal (Figure 3, insert) indicates that two conformations, or two classes of conformations, of the enzyme were present in the Yu et al. preparation: (i) those molecules with a conformation that exhibits the $g' = 12$ EPR signal and only slowly binds cyanide to give the cyanocytochrome a_3 EPR signal in the presence of NO and (ii) those molecules with a conformation that *readily* binds cyanide and reveals a cyanocytochrome a_3 EPR signal in the presence of NO. This result allows the intensity of the $g' = 12$ EPR signal to be quantitated at each time point in Figure 3 by assuming that the intensity of the $g' = 12$ EPR signal is proportional to the fraction of enzyme molecules unaccounted for by the NO-induced cyanocytochrome a_3 EPR signal. It was in this manner that the proportionality constant in eq 1 was determined (Figure 3, insert). We have used eq 1 to estimate the fraction of enzyme molecules that exhibit the $g' = 12$ EPR signal in the preparations as isolated. The fraction of the enzyme molecules in which NO uncouples Cu_{a_3} from cytochrome a_3 was also estimated from the intensity of the NO-induced rhombic high-spin cytochrome a_3 EPR signal. These results are shown for both the Hartzell and Beinert and Yu et al. preparations in Table II. The obvious conclusion from the data in Table II is that the sum of the molecules exhibiting the $g' = 12$ EPR signal plus those exhibiting an NO-induced $g = 6$ EPR signal does not account for 100% of the enzyme molecules in the Yu et al. preparation. In fact, this sum was different for the two preparations of the enzyme, accounting for close to 100% of the enzyme molecules in the Hartzell and Beinert preparation but only 75% of the Yu et al. preparation. Thus, in the Yu et al. preparation, there exists

Table II: Intensities of the $g' = 12$ and NO-Induced $g = 6$ EPR Signals in Oxidized Cytochrome *c* Oxidase *Plus* Fluoride

preparation	time of incubation (h)	fraction $g' = 12$	fraction $g = 6$	sum ($g' = 12$ and $g = 6$)
Yu et al. (1975)	0	0.74	0.01 (rhombic)	0.75
	4	0.74	0.20 (axial)	0.94
	16	0.72	0.28 (axial)	1.00
Hartzell & Beinert (1974)	0	0.39	0.56 (rhombic)	0.95
	4	0.45	0.51 (axial)	0.96
	17	0.45	0.52 (axial)	0.97

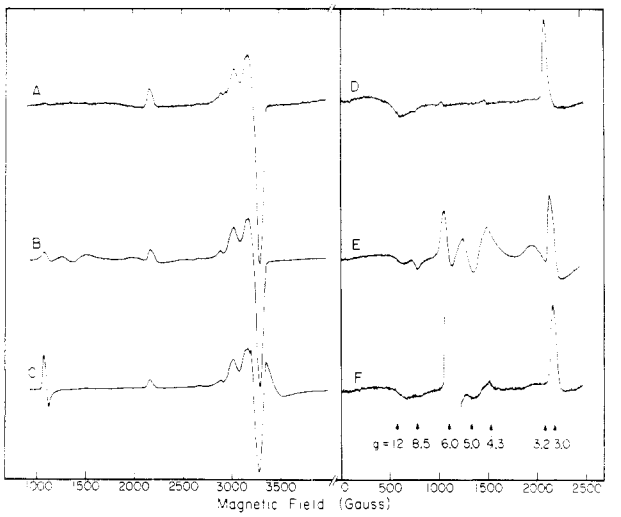

FIGURE 4: EPR spectra of the Yu et al. (1975) preparation of cytochrome *c* oxidase: (A) native anaerobic enzyme; (B) 100 mM KF added to (A) and incubated 24 h at 4 °C; (C) 1 atm of NO added to (B), mixed, and immediately frozen. Spectra D–F are the same as spectra A–C, respectively, with a 4-fold increase in gain. Conditions: temperature, 17 K; microwave power, 0.5 mW; modulation amplitude, 16 G; microwave frequency, 9.24 GHz.

an additional subset of enzyme molecules which exhibits neither the $g' = 12$ EPR signal nor the NO-induced high spin cytochrome a_3 signal but *readily* binds cyanide to give a cyanocytochrome a_3 EPR signal in the presence of NO. Some insight into the nature of this third subset of enzyme molecules has been provided by the results of fluoride binding studies, which we now describe.

Oxidized Enzyme Plus Fluoride and NO. Fluoride was found to bind to cytochrome a_3 while NO was coordinated to Cu_{a_3} (Stevens et al., 1979), revealing an axial high-spin fluoroferricytochrome a_3 EPR signal. We investigated the possibility that fluoride might slowly bind to the conformation that exhibited a $g' = 12$ EPR signal (as did cyanide) and, after long incubation, allow 100% of the fluorocytochrome a_3 EPR signal to be induced by NO.

When fluoride was added to the Yu et al. preparation in the *absence* of NO, a new, unusual EPR signal appeared. The new signal (hereafter referred to as the fluorocytochrome a_3–Cu_{a_3} EPR signal) spanned at least from 800 to 2000 G at X-band and had five resonances with *g* values of 8.5, 6, 5, 4.3, and 3.2 (Figure 4B,E). The addition of NO to the oxidized enzyme–fluoride complex immediately eliminated the fluorocytochrome a_3–Cu_{a_3} EPR signal, and in its place the high-spin fluoroferricytochrome a_3 EPR signal was observed (Figure 4C). This observation demonstrates that the fluorocytochrome a_3–Cu_{a_3} EPR signal is associated with the cytochrome a_3–Cu_{a_3} site. Moreover, the intensity of the NO-induced high-spin fluoroferricytochrome a_3 and the $g' = 12$ EPR signals together accounted for very close to 100% of the enzyme molecules (Table II).

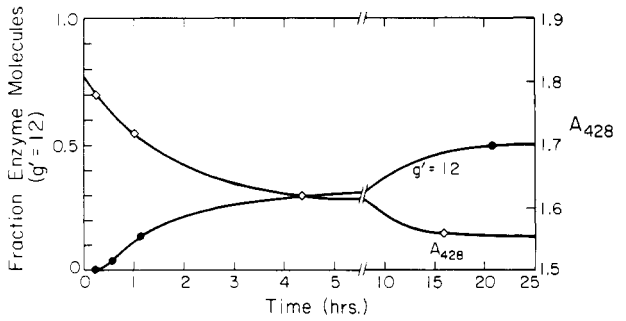

FIGURE 5: Change in absorption at 428 nm and change in the fraction of enzyme molecules which exhibited a $g' = 12$ EPR signal with time after reoxidation of the fully reduced enzyme with air. The Hartzell & Beinert (1974) preparation was used. The concentration of cytochrome *c* oxidase was 0.16 mM (EPR) and 0.02 mM (optical). The EPR conditions were the same as in Figure 2.

For the Hartzell and Beinert preparation, essentially 100% of the enzyme molecules were accounted for in the absence of fluoride by the sum of the intensities of the $g' = 12$ and NO-induced $g = 6$ EPR signals (Table II). In this preparation, fluoride appears to bind quantitatively to that fraction of enzyme molecules in which NO binding induced an EPR signal from cytochrome a_3. There was no indication that fluoride binds at all to the fraction of the enzyme molecules which exhibit the $g' = 12$ EPR signal (Table II).

These observations on the Hartzell and Beinert preparation can be contrasted with those on the Yu et al. preparation. In the Yu et al. preparation, only 75% of the enzyme molecules were accounted for by the sum of the intensities of the $g' = 12$ and NO-induced $g = 6$ EPR signals (Table II). Yet, in the presence of *both* NO and fluoride, 100% of the enzyme molecules could be accounted for. It appears then that for a fraction of the oxidized enzyme in the Yu et al. preparation NO *alone* did not induce a cytochrome a_3 EPR signal, but NO and fluoride *in combination* did.

The third conformation implicated in the Yu et al. preparation may be related to the unusual fluorocytochrome a_3–Cu_{a_3} EPR signal induced by fluoride in the absence of NO. There was no evidence for such a third conformation in the Hartzell and Beinert preparation; fluoride induced very little, if any, of the unusual fluorocytochrome a_3–Cu_{a_3} EPR signal from this preparation (spectra not shown). Thus, it appears that the unusual fluorocytochrome a_3–Cu_{a_3} EPR signal was generated only from a fraction of the oxidized Yu et al. preparation: this subset of enzyme molecules was distinct from the fractions that exhibited the $g' = 12$ EPR signal and the NO-induced $g = 6$ EPR signal.

Reoxidation of the Reduced Enzyme. We have monitored the time evolution of the reoxidized enzyme by EPR and optical spectroscopy. The time scale of our experiments was, however, sufficiently long that the transient oxidized state previously studied by Shaw et al. (1978) (with characteristic EPR signals at $g = 5$, 1.8, and 1.7) could not have been detected. Thus, the initial species that we observed can be identified as the oxygenated enzyme, using the nomenclature described in the introduction.

The oxygenated enzyme did not exhibit an EPR signal at $g' = 12$ at the outset. However, upon incubation at 4 °C, the $g' = 12$ EPR signal gradually increased in intensity, and the rate at which the $g' = 12$ EPR signal appeared paralleled the rate at which the Soret band at 428 nm shifted to 420 nm (Figure 5). However, even after 2 days of incubation of the reoxidized enzyme at 4 °C, *no* EPR signals from cytochrome a_3 were induced by NO.

The addition of fluoride to the oxygenated enzyme was found to induce a *large* fluorocytochrome a_3–Cu_{a_3} EPR signal. Moreover, the addition of NO to the fluoride-bound oxygenated enzyme induced a high-spin fluoroferricytochrome a_3 EPR signal. Thus, it appears that the oxygenated conformation can be identified as the conformation that accounted for about 25% of the enzyme molecules in the Yu et al. preparation, but which was virtually absent in the Hartzell and Beinert preparation.

Discussion

Identification of Four Conformations of Oxidized Cytochrome c Oxidase. We have obtained evidence that at least three conformations of oxidized cytochrome *c* oxidase can exist in the enzyme as isolated. These conformations are not in rapid equilibrium; hence, it was possible to distinguish them on the basis of their EPR properties. The sum of these conformations accounted for 100% of the enzyme molecules in both the Hartzell and Beinert and the Yu et al. enzyme preparations examined in this work. These conformations were also found to account for all the enzyme molecules in a second Hartzell and Beinert preparation, the results of which we have not described here. However, the fraction of the enzyme molecules in each conformation did vary among these preparations. For two Hartzell and Beinert preparations in which the activity of the enzyme was substantially lower than the optimum [$\leq$60 mol of cytochrome *c* (mol of cytochrome *c* oxidase)$^{-1}$ s^{-1}] (Vik & Capaldi, 1980), we found that the three conformations (resting, *g*12, and oxygenated) accounted for less than 100% of the enzyme molecules present. We attribute this discrepancy to the presence of inactive enzyme molecules.

The most important conclusion that has emerged from the present work is that when the reduced enzyme is reoxidized with O_2 the oxidized enzyme rapidly relaxes from the *g*5 conformation into the oxygenated conformation, after which a percentage of the enzyme molecules slowly relaxes into the *g*12 conformation. Thus, two of the conformations that we have distinguished can be identified as states formed upon reoxidation of the reduced enzyme with O_2. However, the resting conformation is not formed when the enzyme is reoxidized with O_2. We now discuss these various conformations in turn.

Resting Conformation. The resting conformation has only been observed in the enzyme as isolated and never appeared after the enzyme was passed through a cycle of reduction and reoxidation. Perhaps a step in the isolation of the enzyme transformed a fraction of the enzyme molecules into the resting conformation and, thereafter, this conformation remained until the enzyme was reduced and reoxidized. In this regard, reoxidation of the reduced enzyme by ferricyanide in the *absence* of O_2 did not regenerate any of the resting conformation.

The resting conformation was distinguished by a high-spin cytochrome a_3 EPR signal that was induced [see Figure 1B in Stevens et al. (1979)] by binding of NO. This high-spin cytochrome a_3 EPR signal observed in the presence of NO ($g = 6.16$, 5.82, and 2) has also been observed by Rosén et al. (1977) during partial reduction of oxidized cytochrome *c* oxidase in the *absence* of NO. Rosén et al. (1977) found that the conformation of the enzyme exhibiting this EPR signal did not rapidly react with O_2 when both O_2 and reductant were added to the oxidized enzyme. They concluded that this conformation was associated with the resting state of the enzyme. In view of the observations by Rosén et al. (1977), we identify the conformation that exhibits a cytochrome a_3 EPR signal in the presence of NO as the resting conformation. This assignment is in accord with the observation of Antonini et al. (1977) that cytochrome *c* oxidase can exist in two classes of "conformations". One reacts rapidly with O_2 upon the

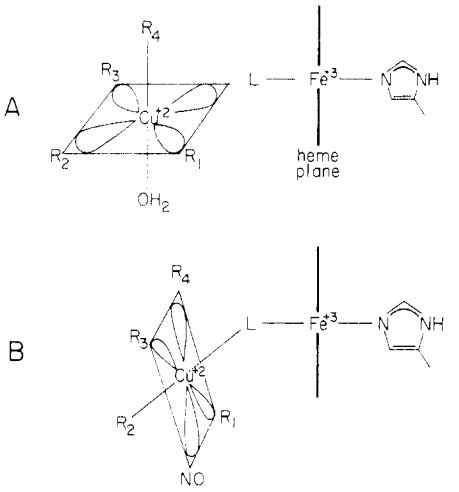

FIGURE 6: Model of the *oxidized* cytochrome a_3–Cu_{a_3} site proposed by Chan et al. (1980) on the basis of NO-binding studies of cytochrome *c* oxidase. For Cu(II) in tetragonal crystal field, as shown, the unpaired electron resides in a $3d_{x^2-y^2}$ orbital which is depicted by the lobes pointing toward the ligands in the square plane. Here R_1, R_2, R_3, and R_4 denote endogenous ligands to Cu_{a_3} and L denotes the ligand bridging between Cu_{a_3} and cytochrome a_3 which may or may not be an endogenous ligand. This model is appropriate for the "resting" conformation of the oxidized enzyme.

simultaneous addition of both O_2 and reductant and is called the "pulsed" enzyme. Another does not react rapidly with O_2 and reductant and is called the "resting" enzyme. However, it is probable that the conformation called the pulsed enzyme by Antonini et al. (1977) is heterogeneous, possibly including two of the conformations that we have identified in this study.

NO alone uncouples Cu_{a_3} from cytochrome a_3 only in the resting conformation. Chan et al. (1980) interpreted this result in terms of a change in the crystal field around the Cu_{a_3} site. In the absence of exogenous ligands, the resting conformation does not exhibit an EPR signal from the cytochrome a_3–Cu_{a_3} site. Chan et al. (1980) proposed that the lack of EPR signals from the cytochrome a_3–Cu_{a_3} site in the resting conformation was the result of a strong antiferromagnetic exchange interaction (Tweedle et al., 1978) mediated between the two metal centers by a bridging ligand. This bridging ligand, L, was proposed (Chan et al., 1980) to be an axial ligand of cytochrome a_3 and an equatorial ligand of Cu_{a_3} (Figure 6A). The binding of NO to an available axial binding site on Cu_{a_3} can reorient the crystal field of Cu_{a_3} as in Figure 6B, provided the crystal field of R_1, R_3, R_4, and NO is stronger than that of R_1, R_2, R_3, and L. Such reorientation of the crystal field of Cu_{a_3} would place the $3d_{x^2-y^2}$ orbital of Cu_{a_3} in a square plane containing the coordinated NO, thus eliminating or greatly reducing the exchange interaction between Cu_{a_3} and cytochrome a_3, but allowing for a favorable interaction between Cu_{a_3} and the bound NO. Hence, if this picture is correct, it can be concluded that the bridging ligand, L, does not strongly influence the crystal field of Cu_{a_3} in the resting conformation.

The question remains as to the identity of the bridging ligand L in the resting conformation. Our results indicate that this ligand is bound to cytochrome a_3 less strongly than cyanide or fluoride (see Table I). Among the possible candidates for L are water, hydroxyl, carboxylate, and tyrosinate.

Nature of the g′ = 12 EPR Signal. We have identified the *g*12 conformation by an EPR signal at $g' = 12$. The question remains as to the origin of this unusual EPR signal. Magnetic suceptibility measurements on oxidized cytochrome *c* oxidase indicate that cytochrome a_3 and Cu_{a_3} together form an $S = 2$ site (Tweedle et al., 1978). Since all the preparations of the enzyme that we have studied contained a large fraction of the

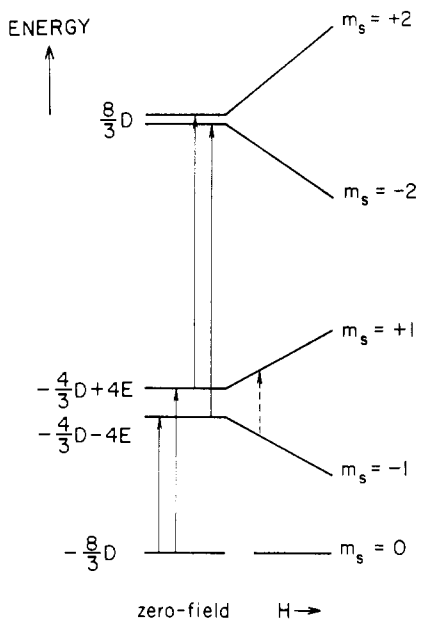

FIGURE 7: The energy levels for an $S = 2$ state formed from the antiferromagnetic coupling of an $S = {}^5/_2$ heme and an $S = {}^1/_2$ copper. *D* and *E* refer to the axial and rhombic zero-field splittings and *H* refers to the external magnetic field. The solid lines represent $\Delta m_s = 1$ transitions and the dashed line represents the "allowed" $\Delta m_s = 2$ transition. The energy levels were calculated by Griffith (1971). The spacing between the levels is not drawn to scale.

enzyme molecules in the *g*12 conformation (from 30% to 75%), it must be concluded that the $g' = 12$ EPR signal arises from an $S = 2$ state.

Griffith (1971) has calculated the energy levels for an antiferromagnetically coupled high-spin ferriheme and a cupric copper for the limits when E/D and D/J are small, where *D* and *E* are the axial and rhombic zero-field splittings and *J* is the isotropic superexchange interaction. These energy levels are shown in Figure 7. It is possible to observe four "allowed" $\Delta m_s = 1$ transitions from an $S = 2$ state (solid arrows in Figure 7). However, for systems containing a high-spin heme, the zero-field splitting, *D*, is expected to be 2 cm^{-1} or more. Since conventional EPR spectrometers are designed to operate at 2–35 GHz (0.07–1.1 cm^{-1}) with the magnetic field scanned from 0 to 15 000 G, none of the "allowed" transitions would be observed. However, it should be possible to observe the $m_s = -1$ to $m_s = +1$ transition (dotted arrow in Figure 7). This so-called "forbidden" $\Delta m_s = 2$ transition is analogous to the half-field transition from a triplet state and is allowed for low magnetic fields and when H_1 (the microwave magnetic field) is parallel to H_0 (the static magnetic field) (Wertz & Bolton, 1972).

We have extended the calculations of Griffith (1971) to include cases where E/D and D/J are not small (S. I. Chan, unpublished results). By definition $|E/D|$ can only vary from 0 to 1/3, but there are no limits on D/J. However, the magnetic susceptibility results (Tweedle et al., 1978) require that $-J \geq 200$ cm^{-1}, and the zero-field splitting induced by the high-spin heme is expected to be larger than 2 cm^{-1}. Within these limits, it was found that the only X-band EPR transition from the $S = 2$ state that could occur at $g' = 12$ was the $m_s = -1$ to $m_s = +1$ transition.

It has been observed that the $g' = 12$ EPR signal observed at X-band does not occur at $g = 12$ when the EPR spectrum is recorded at Q-band (W. E. Blumberg, personal communication). We note that the $m_s = \pm 1$ levels for an $S = 2$ species are split in zero field by an energy of $8E$ (Figure 7). When a sufficiently high magnetic field is applied, the $m_s =$

$\pm$ 1 levels will be further split by an energy of $2g_e\beta H_0$, where g_e is the free electron g value (it is assumed here that the $S = 2$ state has an isotropic electronic g value close to the free electron g value), β is the Bohr magneton, and H_0 is the external magnetic field. With the Zeeman interaction included, the EPR transition will occur at

$$h\nu = 2(\tfrac{1}{6}E^2 + g_e^2\beta^2H_0^2)^{1/2} \quad (2)$$

When the transition is measured at constant frequency by varying the magnetic field, the apparent g value, g', is obtained by setting $h\nu = g'\beta H_0$. Rearranging eq 2, one obtains

$$4(g_e/g')^2 = 1 - 64E^2/(h^2\nu^2) \quad (3)$$

from which E can be calculated, given the apparent g value. Actually, since at X-band (9 GHz) the transition occurs at $g' = 12$, first-order theory is inadequate. We have undertaken an exact calculation for this problem. Using the apparent g value observed at X-band, we obtained $E = 0.036$ cm^{-1}. Equation 3 should be adequate when this transition is observed at Q-band (35 GHz), and we predict for $E = 0.036$ cm^{-1} that the transition will occur at $g' = 4.1$. Thus, the observation by Blumberg that the $g' = 12$ EPR signal observed at X-band changes its apparent g value when the EPR signal is recorded at other frequencies is consistent with the assignment of this signal to a $\Delta m_s \pm 2$ transition from an $S = 2$ state.

g12 Conformation. The above considerations indicate that cytochrome a_3 and Cu_{a_3} together form an $S = 2$ site in the $g12$ conformation and that cytochrome a_3 has nearly axial symmetry (E is close to 0). The ligand binding properties of the $g12$ conformation suggest that cytochrome a_3 is six-coordinate with neither axial ligand being readily dissociable. Cyanide binds very slowly to this conformation ($t_{1/2} = 45$ min when the HCN concentration is about 2 mM), while fluoride does not appear to bind at all.

Further information on the structure of the $g12$ conformation is provided by the lack of effect by NO on the $g' = 12$ EPR signal. This result indicates that either (i) NO does not bind to Cu_{a_3} as in Figure 6 or (ii) NO can bind to Cu_{a_3} but does not uncouple Cu_{a_3} from cytochrome a_3 in the $g12$ conformation.

Cyanide slowly binds to cytochrome a_3 in the $g12$ conformation and, once bound, the $g' = 12$ EPR signal is eliminated. However, no other EPR signals are observed from the cytochrome a_3–Cu_{a_3} site in the presence of cyanide. This result suggests that cyanide facilitates an antiferromagnetic coupling between Cu_{a_3} and cytochrome a_3 when bound. In this regard, magnetic susceptibility measurements (Tweedle et al., 1978) indicate that Cu_{a_3} and cytochrome a_3 are antiferromagnetically coupled in the oxidized cyanide-bound enzyme with an exchange interaction of 40 cm^{-1}. The interesting observation, though, is that NO readily uncouples Cu_{a_3} from cytochrome a_3 once cyanide has bound to cytochrome a_3.

This observation can be explained as follows. When cyanide binds to cytochrome a_3 it replaces the ligand bridging between cytochrome a_3 and Cu_{a_3} (L in Figure 6). In this configuration, the $3d_{x^2-y^2}$ orbital of Cu_{a_3} is directed toward the bridging cyanide ligand and an antiferromagnetic exchange interaction of 40 cm^{-1} is facilitated by cyanide between the two metals. We surmise that NO coordinates to the free axial position on Cu_{a_3} and rearranges the crystal field about Cu_{a_3} such that one lobe of the $3d_{x^2-y^2}$ orbital of Cu_{a_3} now is directed toward the coordinated NO. The question then is: to which of the remaining ligands do the lobes of the Cu_{a_3} $3d_{x^2-y^2}$ orbital point? This will be determined by the crystal-field strength of R_1 and R_3 relative to that of R_2 and the bridging cyanide (Figure 6).

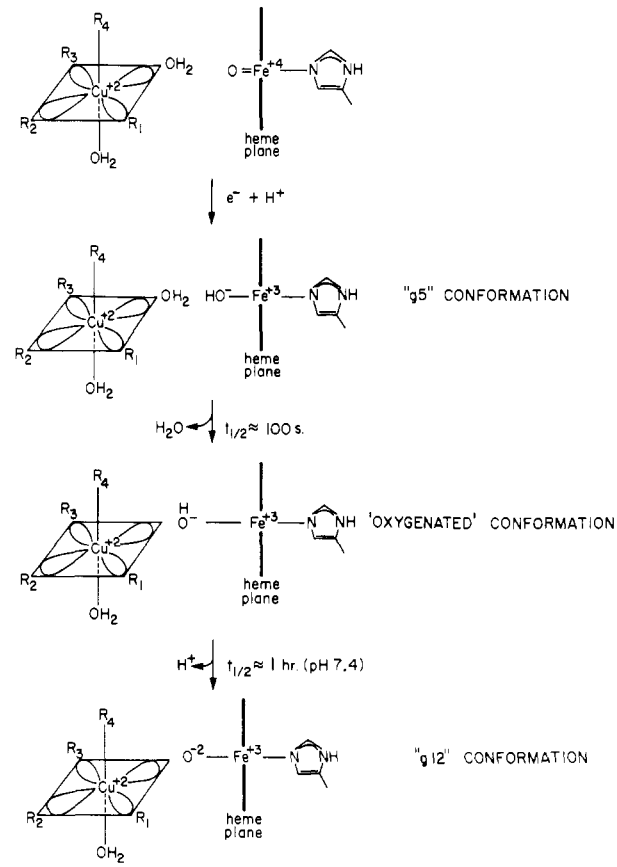

FIGURE 8: Proposed sequence of states formed upon reoxidation of reduced cytochrome c oxidase by O_2. In all cases, Cu_{a_3} is proposed to have a tetragonal structure. For a tetragonal Cu(II), the unpaired electron resides in a $3d_{x^2-y^2}$ orbital which is depicted by the lobes pointing toward the ligands in the square plane. R_1, R_2, R_3, and R_4 denote endogenous ligands.

Since in the absence of exogenous ligands the $3d_{x^2-y^2}$ orbital is directed toward R_1, R_2, and R_3, these three ligands must provide a reasonably strong crystal field. However, Fe(III)–CN^- is not a particularly strong field ligand; hence, it is quite reasonable that the coordination of NO to the axial position of Cu_{a_3} would rearrange the crystal field about Cu_{a_3} such that the $3d_{x^2-y^2}$ orbital no longer points toward the ligand bridging between the two metals when the bridging ligand is cyanide. In this manner, the mechanism by which NO uncouples Cu_{a_3} from cytochrome a_3 in the presence of cyanide may be understood.

The above considerations also suggest that the difference between the $g12$ conformation and the resting conformation (Figure 6) may lie in the nature of the ligand that bridges the two metals. For example, if the bridging ligand in the $g12$ conformation is both a *strong-field* ligand of Cu_{a_3} and *strongly bound* to cytochrome a_3 and Cu_{a_3} whereas the bridging ligand in the resting conformation is a *weak-field* ligand of Cu_{a_3} and is *weakly bound* to cytochrome a_3, then the differences between these two conformations could be explained. It should be noted that the bridging ligand in both of these conformations must mediate a strong antiferromagnetic exchange interaction between the two metal centers.

In view of the above considerations, we proposed the model for the $g12$ conformation of the oxidized enzyme shown in Figure 8. In this model, cytochrome a_3 has both axial positions occupied by strongly bound ligands, a histidine and a μ-oxo ligand, and, hence, it would not be expected to readily bind exogenous ligands. The strong antiferromagnetic exchange interaction between Cu_{a_3} and cytochrome a_3 in this

conformation would be mediated through the μ-oxo bridge.

It has in fact been suggested that a reasonable structure in which Cu_{a_3} and cytochrome a_3 can be strongly antiferromagnetically coupled with $-J > 200$ cm^{-1} is one in which Cu_{a_3} and cytochrome a_3 are bridged by a μ-oxo ligand (Blumberg & Peisach, 1979). Such a structure is attractive because such a bridge may easily be formed upon the four-electron reduction of O_2.

Nature of the Fluorocytochrome a_3–Cu_{a_3} EPR Signal. When fluoride is added to the oxygenated conformation, an unusual EPR signal is induced with resonances at $g = 8.5$, 6, 5, 4.3, and 3.2, which we have referred to as the fluorocytochrome a_3–Cu_{a_3} EPR signal. This signal must arise from a state in which both cytochrome a_3 and Cu_{a_3} are oxidized, since no reductant was added to the enzyme; moreover, the signal was immediately eliminated by NO without the appearance of any EPR signals characteristic of the partially reduced NO-bound enzyme [see Brudvig et al. (1980)].

When two paramagnetic sites are in close proximity, it is expected that both exchange and dipolar interactions will greatly modify the EPR spectra of both sites (Smith & Pilbrow, 1974). In fact, a strong exchange interaction can completely eliminate the EPR signals from either site, such as occurs when two $S = {}^1/_2$ sites are strongly antiferromagnetically coupled. In cytochrome *c* oxidase, we have an $S = {}^5/_2$ heme interacting with an $S = {}^1/_2$ copper. As we noted earlier in our discussion of the $g' = 12$ EPR signal, multiple EPR resonances are not expected at X-band for a strongly antiferromagnetically coupled heme and copper. We must, therefore, conclude that the fluorocytochrome a_3–Cu_{a_3} EPR signal arises from a state in which Cu_{a_3} and cytochrome a_3 are weakly exchange and/or dipolar coupled. Thus, fluoride must largely eliminate the exchange interaction between Cu_{a_3} and cytochrome a_3 upon binding to cytochrome a_3 in the oxygenated conformation.

Reduced Enzyme with O_2: The g5 and Oxygenated Conformations. When reduced cytochrome *c* oxidase is reoxidized with O_2, three conformations are sequentially formed: first, the *g*5 conformation; second, the oxygenated conformation; third, the *g*12 conformation. The structure that we have proposed for the *g*12 conformation suggests a sequence by which these conformations can be formed upon the reaction of the reduced enzyme with O_2.

Brudvig et al. (1980) recently proposed a mechanism for the reaction of O_2 with reduced cytochrome *c* oxidase. This mechanism included generation of an iron(IV) oxide state after the enzyme had transferred three electrons to the coordinated O_2. The transfer of the fourth electron to this iron(IV) oxide site converted the enzyme into the fully oxidized enzyme. If this mechanism for the reaction of O_2 with the reduced enzyme is correct, then a series of transient states should be formed after the iron(IV) oxide state is reduced with another electron in order to account for the observations reported here. A possible scheme is depicted in Figure 8. (1) Initially an iron(III) oxide state would be formed. (2) The iron(III) oxide would be rapidly protonated to form an iron(III) hydroxyl. (3) Then a ligand, presumably water, would have to be displaced from Cu_{a_3} to permit formation of a hydroxyl bridge between Cu_{a_3} and cytochrome a_3. (4) Finally, Cu_{a_3} and cytochrome a_3 would have to move closer together, probably mediated by the protein matrix, to form the proposed μ-oxo bridge. We propose that the *g*5, oxygenated, and *g*12 conformations can be identified as three of the intermediates formed in sequence upon reoxidation of the reduced enzyme by O_2.

The *g*5 conformation exhibits an unusual EPR signal in the absence of added ligands. If Cu_{a_3} and cytochrome a_3 are not bridged by a common ligand, as in the structure for the *g*5 conformation shown in Figure 8, then these two metal centers would not be strongly exchange coupled, but rather weakly exchange and/or dipolar coupled. At this time, it is not clear whether the EPR signal from the cytochrome a_3–Cu_{a_3} site in the *g*5 conformation can be accounted for by a dipolar-coupled high-spin ferricytochrome a_3 and $Cu_{a_3}{}^{2+}$.

The oxygenated conformation does not exhibit any EPR signals from the cytochrome a_3–Cu_{a_3} site at X-band in the absence of added ligands but does exhibit an unusual fluorocytochrome a_3–Cu_{a_3} EPR signal in the presence of fluoride. These observations can be explained if a hydroxyl bridge facilitates an antiferromagnetic exchange interaction between cytochrome a_3 and Cu_{a_3} in the absence of added ligands and if fluoride readily displaces the hydroxyl bridge from cytochrome a_3, leaving a dipolar-coupled $Cu_{a_3}{}^{2+}$–OH^-, fluoroferricytochrome a_3 site.

Implications for Previous Work. We have shown that oxidized cytochrome *c* oxidase can exist in three conformations (*g*12, oxygenated, and resting) in addition to the transient *g*5 conformation first formed upon reoxidation of the reduced enzyme. The enzyme as isolated was found to contain variable proportions of the *g*12, resting, and oxygenated conformations. The presence of more than one conformation of the enzyme as isolated creates a substantial problem in the interpretation of physical studies of the oxidized enzyme. Each conformation appears to have different ligand binding properties, and it is probable that these conformations are different with respect to the kinetics of reaction with reductant and O_2 [as already noted by Antonini et al. (1977)], optical spectra, magnetic susceptibility, and redox properties. The optical changes observed with time when the oxidized enzyme is mixed with a reductant bear directly on this problem. It has been observed that reduction of the enzyme by dithionite is biphasic (Lemberg & Mansley, 1965; Petersen & Cox, 1980). This observation was interpreted as the rapid reduction of cytochrome *a* followed by a slow reduction of cytochrome a_3. We feel these data can be equally well explained by the rapid reduction of both hemes in some of the conformations of the enzyme and the slow reduction of both hemes in the remaining conformations. The resolution of this point is important, since studies of this type have been used to deconvolute the optical spectrum of cytochrome *c* oxidase into the spectra of the component hemes, namely, cytochromes *a* and a_3.

This work should provide impetus for the development of techniques for preparing homogeneous samples of oxidized cytochrome *c* oxidase. Only then can the optical spectra, magnetic circular dichroism, magnetic susceptibility, and the structure of each conformation become more precisely defined than is currently possible.

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