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THE OXYGEN REACTIONS OF REDUCED CYTOCHROME *c* OXIDASE

POSITION OF A FORM WITH AN UNUSUAL EPR SIGNAL IN THE SEQUENCE OF EARLY INTERMEDIATES

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Summary

The order of appearance of intermediates in the reoxidation of reduced cytochrome *c* oxidase by oxygen has been examined. Particular emphasis was placed on determining where the intermediate with the EPR signal at $g = 5$, 1.78, 1.69 (Shaw, R.W., Hansen, R.E. and Beinert, H. (1978) J. Biol. Chem. 253, 6637—6640) appears in the sequence of events during reoxidation. Flash photolysis of reduced, CO-complexed samples of cytochrome *c* oxidase in the presence of oxygen in a buffer containing 30% (v/v) ethylene glycol at 77 K and 195 K has been used to generate states of partial reoxidation. The intermediate with the EPR signal at $g = 5$, 1.78, and 1.69 can be detected as a product of the photolysis and subsequent oxidation but does not appear until the photolyzed sample is incubated at temperatures well above 195 K. In the course of the reoxidation, the intermediate characterized by the $g = 5$, 1.78, 1.69 signal occurs in the reaction sequence after the states referred to as 'Compound A' and 'Compound B' (Chance, B., Saronio, C., and Leigh, J.S. (1975) J. Biol. Chem. 250, 9226—9237). Its apperance is within the time range reported for the formation of 'oxygenated' cytochrome *c* oxidase (Orii, Y. (1979) in Cytochrome Oxidase (King. T.E., Orii, Y., Chance, B. and Okunuki, K., eds.), pp. 331—340, Elsevier/North-Holland Biomedical Press, Amsterdam).

Introduction

We have recently reported the observation by EPR spectroscopy of a transient species of cytochrome *c* oxidase (EC 1.9.3.1) which is generated by

rapid reoxidation of the reduced enzyme by oxygen and have described and discussed some of the properties of this oxidized form of the enzyme including its novel EPR signal which appears at $g = 5$, 1.78, 1.69 [1]. Evidence has accumulated from a number of laboratories that cytochrome *c* oxidase can exist in several oxidized forms such as the anaerobically reoxidized form [2], the forms collectively called 'oxygenated' oxidase [3—6] and activated, oxidized forms [7—9]. The study of these various states is of interest because it has been shown that such forms are in general more reactive than the form obtained by the usual purification procedures [1,7—11]. In spite of the inherent difficulty of comparing the multitude of optical spectroscopic data reported for the 'oxygenated' species to that for the species we observed, it seems clear that the time scale for the appearance of these forms are similar. In fact it is likely that the form we observe is the activated form described by Antonini et al. [7] and Brittain and Greenwood [8] and perhaps is also a component of or even the essential species of 'oxygenated' oxidase [1,3—6]. Until now, however, the relationship of our species to those which arise presumably on the μs timescale at room temperature and to date have been trapped only at subzero temperatures in aqueous/organic solvent mixtures [12,13] had not been explored. We have been able to clarify that relationship with the experiments described herein.

The purpose of these experiments is to establish the chronological order of the appearance of the species we have identified by EPR spectroscopy of $g = 5$, 1.78, 1.69 relative to that of the species observed by difference spectroscopy during the reoxidation of reduced cytochrome *c* oxidase by Chance and coworkers [12,13]. We have followed an entirely empirical approach, attempting to reproduce the reported results and to locate our intermediate in the sequence of events without entering the discussion of their chemical interpretation or catalytic significance [12—17].

Materials and Methods

Purification of cytochrome *c* oxidase from beef heart mitochondria, rapid mixing and freezing, and EPR and low-temperature optical reflectance spectroscopies were carried out as described previously [1,2,11,18,19]. The buffer used was 5 mM Tricine and 5 mM sodium cacodylate containing 0.2% (w/v) Tween 20 and, unless otherwise specified, 30% (v/v) ethylene glycol (Fisher Scientific), all at an apparent pH at 25°C of 7.2. Cytochrome *c* oxidase concentrations are expressed in terms of total heme *a* present. The NADH used was obtained from P-L Laboratories. Pigeon heart mitochondria were prepared by the method of Chance and Hagihara [20] in a medium of 0.225 M mannitol, 0.075 M sucrose, 0.2 mM EDTA, and 0.05 M Tris at pH 7.4. Oxygen which was 90% enriched in ^{17}O was obtained from Miles Laboratories.

In the preparation of all reduced samples of cytochrome *c* oxidase, either for flash photolysis or rapid reaction experiments, whether or not CO was present, the reduction was carried out using an excess of NADH in the presence of a small quantity of cytochrome *c*. Since reduction by this method requires several hours, there is no complication of reduction by the excess NADH when the reaction with oxygen occurs later.

Samples for flash photolysis were prepared using the demountable evacuation vessels and EPR tubes described previously [21]. Enzyme, cytochrome *c*, buffer without ethylene glycol, and NADH were added to one side-arm of the vessel, while sufficient 100% ethylene glycol to make the final mixture 30% (v/v) in ethylene glycol was added to the other. The vessel was evacuated and flushed repeatedly and left under vacuum. All subsequent operations were performed in the dark. Purified CO [21] was admitted and the reduction in the aqueous enzyme solution was allowed to proceed at 2°C in the dark under the CO atmosphere for 24 h. Then, the enzyme solution was mixed with the ethylene glycol, the mixture (about 150 μl) was transferred to the bottom of the tube, and the tube was placed in a cooled ethanol bath at −15°C for 5 min. Next, the demountable evacuation vessel was detached and approximately 500 μl of oxygen-saturated buffer at −15°C was added to the tube and quickly stirred into the protein solution in the ethanol bath. The sample was frozen in less than 30 s in liquid nitrogen and stored in an aluminum foil wrapper in the dark at 77 K until used for flash photolysis. The final concentrations of cytochrome oxidase, cytochrome *c* and NADH were typically 200—230 μM, 10—12 μM, and 22—25 mM, respectively. Samples prepared by this method were largely reduced; however, the appearance of approximately 26—32% of the maximal EPR-detectable copper signal and as much as 3—7% of the maximal low-spin heme signal at $g = 3$ prior to photolysis indicated that during the preparation procedure and handling, a small fraction of the enzyme molecules become reoxidized. Oxidation could have occurred as a consequence of accidental exposure of the sample to stray light or temperature variations. Therefore, these considerations of oxidation state were taken into account in calculating the amount of increase in a given EPR signal during the oxygen reaction. Incubation of a control sample in the dark at −78°C without flashing showed a small, slow increase in the $g = 4.3$, the $g = 3$ heme and detectable copper signals which ceased after 5 min incubation. This increase accounted for about 7% of the maximum $g = 3$ signal and about 8% of the maximum possible detectable copper signal.

Control samples of the reduced enzyme under nitrogen and of the reduced, CO complex for comparisons to optical reflectance spectra were prepared by a similar procedure in the 30% (v/v) ethylene glycol buffer mixture. Because they did not require addition of oxygen-saturated buffer, smaller additions of enzyme were used to reach the necessary concentration. In preparation of these samples, special precautions were necessary to remove oxygen from the viscous ethylene glycol, including boiling the stock ethylene glycol under vacuum immediately before use and storing it under argon. Prior to adding the aqueous solution of enzyme and cytochrome *c* to the side-arm, the correct volume of freshly boiled ethylene glycol was added to the other sidearm as a 60% (v/v) solution and evacuated. Essentially complete reduction was achieved after incubation at 4°C for 48 h.

The apparatus for flash photolysis included a power transformer which, through a rectifier circuit, was used to charge a series of eight 42 μF high-energy storage capacitors (Capacitor Specialists Inc.) to a final voltage of 4 kV DC. The remaining components consisted of a type FX-94C helical xenon flash tube with an effective arc length of 18 inches and a model TM-11A Trigger

Module (both from EG and G Electro-Optics). The TM-11A was used to provide an external trigger pulse of 30 kV to initiate commutation in the xenon flash tube. Under these conditions, the energy input/flash was 2.7 kJ and the flash duration was approximately 1.7 ms at 33% peak amplitude.

The sample in the quartz EPR tube to be photolyzed was suspended in a cylindrical silvered glass dewar which was filled with either ethanol and solid CO_2 at 195 K or liquid nitrogen. The inside diameter of the dewar was 4.8 cm and a single rectangular window was covered by a Corning 9788 (C.S.4-97) filter which measured 5 × 5 × 0.5 cm in order to reduce the amount of infrared light incident on the sample. The flash tube was situated such that the long axis of the helix faced the dewar window and the total distance from the sample to the front of the flash tube was approximately 6 cm. A semicylindrical foil reflector was located 1 cm behind the flash tube. Those samples which were photolyzed at 195 K were quickly transferred from the ethanol/solid CO_2 bath to liquid nitrogen; this required 5—10 s.

Sample incubations above 195 K were carried out under positive nitrogen pressure with the evacuation vessel and stopcock [21] attached to prevent the sample from splitting. When a sample was to be transferred from liquid nitrogen to cooled isopentane at 233 K, it was first quickly immersed in ethanol and solid CO_2 at 195 K for approximately 5 s and then transfered to the 233 K bath for the desired length of time.

A calibrated copper-constantan thermocouple with a Tektronix type 547 oscilloscope were used in a control experiment to measure the temperature rise from 195 K during the flash. The thermocouple was immersed in 300 μl of 1.2 mM cytochrome oxidase, frozen into the sample in a quartz EPR tube and the sample was flashed under the conditions described above. A photograph of the oscilliscope trace showed that the maximum temperature increase (approximately 500 ms after the flash) was 1.6°C.

An additional, double-flash control experiment similar to that described by Chance and coworkers [12] was performed to assess the photolysis efficiency. Flashing the reduced, CO-inhibited enzyme as above at 195 K, quenching the oxygen reaction in liquid nitrogen, returning the sample to 195 K and repeating the procedure for a total incubation at 195 K of 26 s gave no different spectroscopic results than a sample flashed only once and incubated for that period.

Finally, another control experiment was performed to assess the effects of ethylene glycol on the appearance of the EPR signal at g = 5, 1.78, 1.69. In this case, 1.25 mM cytochrome oxidase was reduced, as we have described, with NADH [1] and rapidly mixed for 6 ms at 20°C with oxygen-saturated buffer containing 60% (v/v) ethylene glycol so that the final ethylene glycol concentration after mixing was 30% (v/v). This sample was compared to another obtained in the same manner but using no ethylene glycol.

Results

The control experiment of Fig. 1A and B shows clearly that full development of the g = 1.78, 1.69 resonances of the EPR signal of 'oxygenated' oxidase is unaffected by the presence of 30% (v/v) ethylene glycol in the

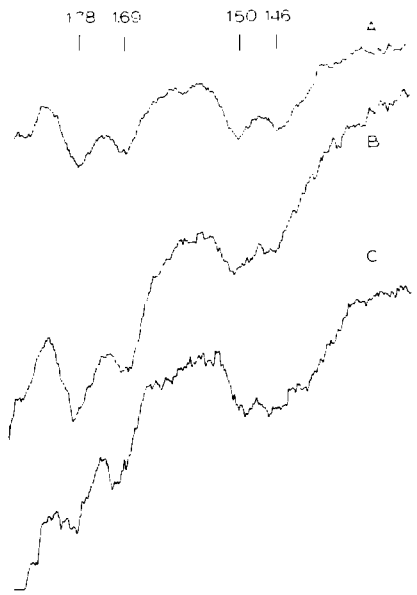

Fig. 1. High-field EPR spectra of rapidly reoxidized cytochrome *c* oxidase in the presence and absence of 30%(v/v) ethylene glycol and also of aerated pigeon heart mitochondria. (A) 1.25 mM enzyme reduced as described in the text and then rapidly reoxidized by mixing with an equal volume of oxygen-saturated buffer without ethylene glycol at 20°C and freezing after 6 ms. (B) A repeat of (A) except that the buffer contained 30%(v/v) ethylene glycol. (C) Pigeon heart mitochondria sample of Fig. 12C (Ref. 19) which was aerated and frozen after 10 min and contained approximately 50 mg/ml protein. The conditions for EPR spectroscopy were: microwave frequency and power 9.2 GHz and 2.7 mW, respectively; modulation amplitude and frequency 0.8 mT and 100 kHz, respectively; scanning rate 100 mT/min, time constant 0.5 s and temperature 13 K. Field positions of the spectra are given on a scale of *g* factors. Spectra A and B are the average of four scans each (Nicolet 1020) while spectrum C is the average of 16 scans. The amplification for (C) was 2.6 times that used for (A) and (B). Note that the samples in (A) and (B) were diluted 2.1-fold by isopentane.

oxygen-saturated buffer used for reoxidation. The reflectance spectra of Fig. 2A—C do not significantly differ from the spectra of the corresponding states in the absence of 30% (v/v) ethylene glycol. An increase in the signal at $g = 4.3$ which is thought to be due to contaminating high-spin iron compounds that are not in effective electronic communication with the catalytic components of the enzyme was observed in the presence of 30% (v/v) ethylene glycol, even in samples incubated at 195 K in the dark without flashing. This increase is in agreement with previous observations [22,23]. Ethylene glycol at —20°C at this concentration has been reported to inhibit electron transfer from cytochrome oxidase to oxygen by about 20% [24].

For comparison with the experiments on purified oxidase, Fig. 1C shows the high-field resonances of 'oxygenated' oxidase (as well as that of oxidized cytochrome *a*) in an aerated sample of pigeon heart mitochondria. These resonances at $g = 1.80$, 1.70 obviously do not differ greatly in field position from those in the isolated bovine enzyme.

The reflectance spectra of Fig. 2 provide data on the visible absorption bands of cytochrome *c* oxidase in control and flash photolysis experiments. Fig. 2A is the spectrum obtained of the oxidized resting enzyme. Important features to note are the Soret band maximum at about 420 nm, the rather short wide

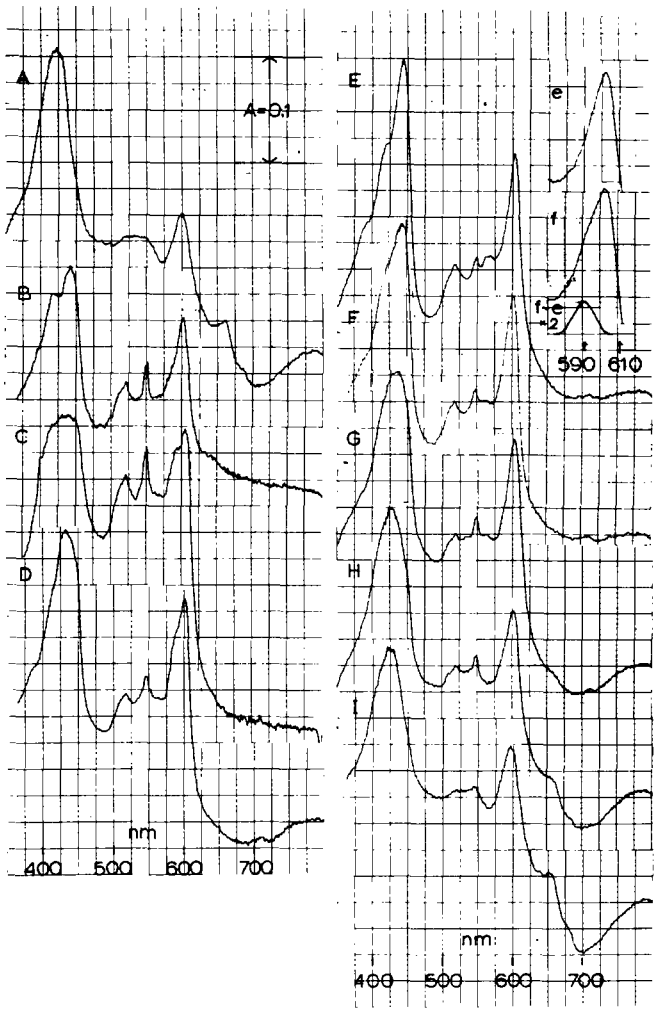

Fig. 2. Optical reflectance spectra of samples at various states of cytochrome *c* oxidase recorded at 97 K. Spectra A—D are of control samples as follows: (A) Oxidized resting enzyme in aerobic buffer; (B) NADH-reduced enzyme in anaerobic buffer; (C) reduced, CO-complexed enzyme in anaerobic buffer; (D) reduced, CO-complexed enzyme containing dissolved O_2 but not flashed. Spectrum E is the result of flash photolysis at 77 K of a sample similar to (D); (F) is the spectrum of the sample in (E) after 129 s incubation at 128 K and 129 s incubation at 158 K. The insets (e) and (f) are the α-peak areas of (E) and (F), respectively, run on 2.5-fold expanded wavelength scale, and a two-fold amplification of the difference spectrum of (f) minus (e) is also shown. Spectrum G is the result of flash photolysis at 195 K of a sample prepared as in (E) and frozen in N_2(l) 6 s after the flash and then incubated for an additional 57 s at 195 K; (H) is the spectrum of the sample from (G) after an addition 1 min incubation at 195 K and 80 s at 233 K; (I) is the spectrum of the sample from Fig. 1B. The enzyme concentration in (A—H) ranged from 200 to 230 μM.

α-band at 600 nm, the fully developed 655 nm band and the near infrared band at about 800 nm which has been attributed to the oxidized copper component(s) [13,25]. Fig. 2B is the spectrum of the anaerobic, reduced enzyme. It shows the position of the reduced Soret bands at about 445 nm, the larger, more slender and symmetrical α-band near 605 nm, and the absence of

appreciable 655-nm and 800-nm bands. The sharp band at 550 nm is due to ferrocytochrome *c* used in the preparation of reduced samples and is also visible to some extent in some of the other spectra of Fig. 2. It has been reported that there is a maximum in the spectrum of reduced cytochrome *c* oxidase near 570 nm which is absent in the reduced CO-complexed form [26]. Indeed this small peak is visible to some extent in some of the spectra of Fig. 2 (e.g. 2E), however, the variable amounts of ferrocytochrome *c* present, and the distortion of absorbance ratios at short and long wavelengths caused by increased scattering losses at shorter wavelengths in reflectance spectroscopy [27], prevent the 570 nm peak from being a completely unambiguous criterion for CO ligation under our conditions. We find the shoulder at 590 nm in the reduced, CO-complexed form to be more reliable for this purpose. An additional complication which may have had an effect on the spectra of Fig. 2B and C, is a difference in the physical state of these samples from any of the others. As was mentioned above, in the preparation of these samples, the ratio of the volume of ethylene glycol to aqueous solution was necessarily greater. Thus the smaller aqueous volume was more susceptible to loss during evacuation and therefore the effective ethylene glycol concentration was likely to be higher than expected. This is the probable explanation for the fact that the samples, whose spectra are shown in Fig. 2B and C were frozen as transparent glasses, unlike any of the other samples whose spectra are shown. Fig. 2C shows the spectrum obtained from the anaerobically reduced, CO-complexed form of the enzyme and it displays the distortions referred to especially at the shorter wavelengths. Nevertheless, important qualitative features are readily discernible, especially the Soret band maximum of about 430 nm and the less symmetrical α-peak which now displays the prominent shoulder at 590 nm, all of which are indicative of CO ligation to reduced cytochrome *c* oxidase. The spectrum in Fig. 2D is that of a sample of the reduced, CO-complexed enzyme to which dissolved oxygen has been added at $-15°$ C in the dark. This spectrum is qualitatively similar to that of Fig. 2C, with the exception that a partially developed 800 nm band is present, reflecting the incomplete reduction of the EPR-detectable copper component referred to in Materials and Methods.

When a reduced, CO-complexed sample which contains dissolved oxygen is flashed, under the conditions described, at 77 K, the resultant spectrum (Fig. 2E) is that of the reduced enzyme in the absence of carbon monoxide (Fig. 2B). The Soret maximum near 445 nm, the slenderness of the α-peak, the complete absence of the 590 nm shoulder and the presence (in this case) of the small band near 570 nm provide further evidence for the completeness of the photolysis of the CO complex. No indication of reoxidation is present in this spectrum nor in the EPR spectrum of this sample.

Incubation of the sample in E for 129 s in a cooled isopentane bath at 128 K and 129 s at 158 K led to spectrum F. Very slight oxidation in the Soret band was observed but this was the only sign of oxidation in the spectrum. However, an increase in the absorption at 590 nm, although not to the state of full development shown in C, was seen. This increase at 590 nm is emphasized on an expanded wavelength scale in Fig. 2e and f which show the α-peak areas of E and F, respectively. A two-fold amplification of the difference spectrum f minus e is also shown; it has a maximum at 590 nm. Following the terminology

of Chance and coworkers, this indicates the formation of 'Compound A' [12,13]. Further incubation of this sample for 10 s at 181 K (not shown) led to the start of reoxidation of the low-spin heme with an increase of 7% of the maximum possible $g = 3$ signal and the detectable copper signal increased by 8% of its maximum. At this point, no EPR signal at $g = 5$, 1.78, 1.69 could be detected.

Flash photolysis as described of a sample of reduced, CO-complexed cytochrome *c* oxidase in the presence of oxygen at 195 K, followed by quenching the sample in liquid nitrogen 6 s after the flash, and further incubation for 51 s at 195 K gave a sample with the spectrum shown in Fig. 2G. As can be seen, some oxidation has occurred in this sample, and in fact, this level of oxidation was reached after only 35 s at 195 K and did not appreciably increase after an additional 51 s at this temperature. Spectrum G shows no shoulder at 590 nm and has some increase in the near-infrared band which can be attributed to oxidation of the copper component(s). Also, there was some slight increase in the 655 nm band, and reoxidation of the a_3 heme was apparent in the Soret region [25]. EPR analysis at this point showed an increase in the $g = 3$ low-spin heme signal of approximately 34% of its maximum, and in the EPR-detectable copper signal of about 32% of its maximum. These changes are consistent with the formation of the species called 'Compound B' [12,13,28,29]. Isolated, solubilized preparations of cytochrome *c* oxidase at the level of 'Compound B' are reported to have a maximum in their difference spectrum (with the spectrum of the reduced enzyme as the reference) at 782 nm [13]. Fig. 2G is consistent with that report. The high-field EPR spectrum of the sample of Fig. 2G is shown as the lower spectrum in Fig. 3. Obviously, the resonances at $g =$ 1.78, 1.69 of 'oxygenated' cytochrome oxidase have yet to appear. The asymmetric bulge on the low-field side of the $g = 1.46$ resonance is due to dissolved oxygen which can cause distortion of the apparent baseline of the spectrum in this region. This was particularly troublesome in samples which contained ethylene glycol.

Fig. 2H shows the spectrum obtained after the sample of Fig. 2G was incubated for an additional 1 min at 195 K and 80 s at 233 K. At this point, the Soret, α-, 655 nm, and near-infrared bands show that considerable oxidation had occurred. EPR analysis indicated that the low-spin heme signal at $g = 3$ had increased by aout 81% of its maximum while the increase in the EPR-detectable copper signal was about 74% of its maximum. The EPR spectrum of this sample at high field is shown as the upper spectrum of Fig. 3. Clearly, the resonances of the intermediate we have observed previously in rapid-mixing experiments are present; their development is approximately 25% of that shown in Fig. 1B. Further incubation of a similar sample at 233 K for 3.5 min did not increase the amount of the $g = 1.78$, 1.69 signal observed. The spectrum of Fig. 2I is that of the sample of Fig. 1B, which has fully developed $g = 1.78$, 1.69 signals; obviously, Fig. 2H and I are very similar.

Finally, Fig. 4 shows a superimposition of the high-field EPR spectra of rapidly reoxidized samples of cytochrome *c* oxidase in aqueous buffer which had been saturated at 16°C with either $^{16}O_2$ or O_2 which was 90% enriched in ^{17}O. Clearly, the two spectra are virtually indistinguishable; no obvious line broadening by this oxygen isotope analogous to that observed for laccase

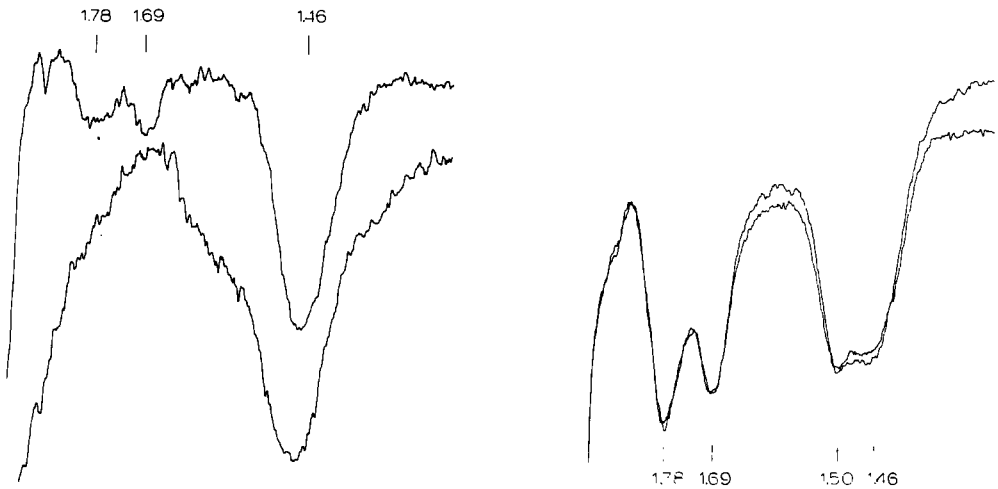

Fig. 3. EPR spectra at high field of sample obtained from flash photolysis of the reduced cytochrome oxidase-CO complex. The upper spectrum is the average of 64 scans of the sample from Fig. 2H, while the lower spectrum is the average of 100 scans of the sample from Fig. 2G. The amplification of the lower spectrum is 1.25 times that of the upper; the conditions of EPR spectroscopy were otherise the same as those of Fig. 1.

Fig. 4. EPR spectra at high field of rapidly reoxidized cytochrome *c* oxidase. As described in the text, 1 mM enzyme was reduced and rapidly reoxidized by mixing with an equal volume of 10 mM sodium cacodylate buffer (pH 7.2) saturated with $^{16}O_2$ or O_2 which was 90% enriched in ^{17}O at 16°C and frozen in isopentane after 6 ms. The EPR conditions were those of Fig. 1C.

[30,31] is seen. However, as we have mentioned previously [1], a weak interaction with oxygen might not be detected.

Discussion

Previous data indicated that full development of the transient species with the EPR resonances at $g = 5$, 1.78, 1.69 could be obtained after a variety of chemical reduction methods prior to reoxidation [1]. It is perhaps not surprising then that this species can also be generated after photolysis of the reduced, CO-complexed enzyme in the presence of oxygen. The fact that some of this intermediate accumulates under these conditions indicates that at least a significant fraction of the enzyme molecules are undergoing the same reactions at subzero temperatures in 30% (v/v) ethylene glycol that we observe in aqueous buffer at room temperature. Thus, while competing reactions in which CO recombines with the enzyme [17] might interfere with the formation of the intermediate and might be an explanation for the fact that the yield of the intermediate is lower than in rapid-reaction experiments, some enzyme molecules obviously are reoxidized by the 'normal' pathway. We have already shown that the transient form decays with time to the oxidized resting enzyme. This observation together with the fact that not all molecules were reduced to begin with could also explain why the yield of the intermediate was not optimal in flash photolysis experiments. It should be noted in this context that few, if any, of the states through which reduced cytochrome *c* oxidase samples pass on reaction with oxygen can be considered as consisting of a single intermediate of

unique electronic structure, rather than a mixture of species. This is quite obvious from the partial appearance or disappearance of optical or EPR features of certain oxidation states of the oxidase components such as in Fig. 2b, which is the spectrum of the state called 'Compound B' [12,13].

The data of Figs. 2 and 3 together with our previous observation and those of others [12—15,22—24,28] establish the following sequence of events after photolysis considering a minimum number of intermediates. First to appear is reduced cytochrome *c* oxidase followed by the states termed 'Compound A' and 'Compound B', respectively. Next to appear is the species with the EPR signal at $g = 5$, 1.78, 1.69 and this is converted to the resting oxidized form, possibly via additional intermediate stages [32]. The appearance of the EPR signal at $g = 5$, 1.78, 1.69 [1] occurs in the time range reported for the formation of 'oxygenated' oxidase [32]. Although no difference between the EPR spectra of oxidized and oxygenated cytochrome *c* oxidase has been observed previously [4], according to our experience, the conditions of EPR spectroscopy and sample preparation used by those authors would not have allowed detection of the EPR signal at $g = 5$, 1.78, 1.69.

Considering the nature of the intermediate and the formal oxidation state of the oxidase components within it, the optical and EPR spectra suggest that cytochromes *a* and a_3 and the EPR-detectable copper are at least 80% oxidized. The appearance of the 655 nm absorption also suggests that the EPR-undetectable copper is oxidized and participating in a spin-coupled state. However, the origin of the 655 nm band is admittedly not fully understood. The results obtained on reoxidation of the enzyme with oxygen enriched with ^{17}O gave no indication that oxygen is involved in the structure. However, this experiment cannot exclude possible weak interactions of oxygen with the metals. As reported previously, it is likely that the EPR spectrum we observe represents a species of spin multiplicity greater than 1/2 [1].

This transient oxidized form of cytochrome *c* oxidase is unique in that it manifests the only EPR signal ($g = 5$, 1.78, 1.69) whose appearance is completely restricted to reoxidation by the natural substrate, molecular oxygen. It therefore deserves serious consideration as an obligatory intermediate in the reoxidation of the reduced enzyme.

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