Biochimica et Biophysica Acta, 722 (1983) 61–71
Elsevier Biomedical Press

BBA 41196

CYTOCHROME *c* OXIDASE

TIME DEPENDENCE OF OPTICAL AND EPR SPECTRAL CHANGES RELATED TO THE 'OXYGEN-PULSED' FORM

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(Received April 21st, 1982)
(Revised manuscript received August 16th, 1982)

Key words: Cytochrome c oxidase; ESR

Time-dependent changes in the optical spectrum (450–920 nm) of cytochrome *c* oxidase, following oxidation with oxygen of the stoichiometrically reduced form, have been investigated and where possible, attempts have been made to correlate our observations with variations in the EPR spectrum over a parallel time course at 2°C. In this regard, particular emphasis has been placed on establishing absorption features related to the presence of EPR resonances at *g* 5, 1.78 and 1.69, which have been tentatively assigned to a spin-coupled state involving cytochrome a_3 and 'EPR-undetectable Cu' (Beinert, H., Shaw, R.W., Dunham, R.W. and Sands, R.H. (1982) in Oxidases and Related Redox Systems (King, T.E., Mason, H.S. and Morrison, M., eds.), Pergamon Press, Oxford, in the press). For optical studies we have used a versatile rapid-scanning spectrophotometer to obtain well resolved spectra down to 2 ms reaction time. Concomitant with the appearance (within 10 ms) of EPR signals at *g* 5, 1.78 and 1.69 is the presence of an enhanced absorption ($\Delta\varepsilon = 0.25$ mM (heme *a*)$^{-1}\cdot$cm^{-1}) at 660 nm, with a trough (relative to following spectra) at 580 nm. In our hands, this feature disappears in a first-order process with a half-life of 46 s at pH 7.2 and 2°C. The effect of this spectral transformation is to decrease considerably the acuteness of the 655 nm absorption band, previously suggested as representing a state of the enzyme in which ferric cytochrome a_3 is coupled to oxidised EPR-undetectable Cu (Beinert, H., Hansen, R.E. and Hartzell, C.R. (1976) Biochim. Biophys. Acta 423, 339–355). This observation can be correlated satisfactorily with a small field shift of the high-field resonances at *g* 1.78 and 1.69 and a broadening at *g* 1.78. Support for this and further correlative assignments arises from parallel experiments using cytochrome *c* oxidase purified via an alternative procedure, which displays different kinetic behavior. Further transformations of the oxidized enzyme are evident through an approx. 10% decrease in absorbance at 600 nm together with small changes centered at 640 and 665 nm (which serve to restore the sharpness of the 655 nm band). The kinetics, as analyzed by the Guggenheim procedure using the absorbance at 597 nm, indicate approx. 50% first-order linearity (half-life 40 min) with additional species contributing at longer times, while over a parallel time course (0–3 h) the EPR resonances at *g* 5, 1.78 and 1.69 virtually disappear. These novel signals can also be seen at a lower

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Abbreviation: Tricine, *N*-tris(hydroxymethyl)methylglycine.

intensity in samples of cytochrome *c* oxidase anaerobically reoxidized by porphyrexide and frozen after a 6 min incubation period at 4°C. This observation, along with the establishment of similar optical changes over the time course of 1 min to 3 h, suggests that aerobic and anaerobic reoxidation produce common forms of the enzyme. Comparison of the *g* 1.78 and 1.69 resonances between samples rapidly aerobically reoxidized in the presence of $H_2{}^{16}O$ and $H_2{}^{17}O$ yielded no evidence for the presence of any labile oxygen ligand (including OH^-, H_2O) in the coordination sphere of the species involved.

Introduction

The existence of multiple forms of oxidized cytochrome *c* oxidase (EC 1.9.3.1) was first indicated by the observations of Okunuki and coworkers [1] some 25 years ago. By bubbling air through solutions of the enzyme reduced with excess dithionite, they demonstrated the formation of a species having the Soret maximum at 426–428 nm (instead of 418 nm as observed for the enzyme obtained upon isolation) and an enhanced α-absorption band somewhat shifted to longer wavelength [1]. This form or (as seems likely) mixture of forms was termed 'oxygenated' cytochrome *c* oxidase in view of the proposed existence of an enzyme-molecular oxygen complex, although it is now generally accepted that such a formulation is incorrect. Throughout continued investigations the designation oxygenated has been retained [2–9] while recent years have witnessed an increasingly complex situation as additional oxidized variations have been reported [10–21]. These include the so-called 'oxygen-pulsed' cytochrome *c* oxidase [11–14], reputedly an early transient form of the O_2-reoxidized enzyme, although the relationship between this and the oxygenated form(s) is not well defined. Interest has been sustained by evidence that oxygenated and related species are functionally more active than the 'resting' form obtained upon normal procedures of purification [11–13].

While the major part of documentation on alternative oxidized forms has stemmed from optical studies, EPR remains a more valuable diagnostic tool, since at least two of the metal components, the low-spin heme and one of the Cu atoms, are detectable in the oxidized form [22]. Consequently, transient EPR resonances at *g* 5, 1.78 and 1.69, generated upon rapid reoxidation by O_2 [19,20], provide a 'label' for at least one of the alternative forms, as does the *g'* approx. 12 signal observed at longer times [18,21]. In addition, reoxidation by nonphysiological reagents such as ferricyanide and porphyrexide generates a form which displays an intense and predominantly rhombic high-spin EPR spectrum and also lacks the prominent optical feature at 655 nm normally seen in oxidized samples [15,16].

A major question that remains is the relationship between the different forms of the oxidized enzyme, and their physiological significance. Sample heterogeneity is clearly a complicating factor and represents a likely explanation for discordance among various investigators [21]. Studies concerning the origin of the transient EPR resonances at *g* 5, 1.78 and 1.69 have indicated that such signals, the lifetime of which is within the time ranges reported for the oxygenated and pulsed forms, arise from the site of O_2 reduction itself [19,20]. In view of our ability to generate this species and monitor simultaneously time-dependent EPR and optical changes, we have undertaken investigations directed at determining which optical features, if any, may be regarded as being characteristic of this state (or states) of the oxidase. With regard to generation of the EPR signals at *g* 5, 1.78 and 1.69, we have also carried out experiments with the aim of reconciling with our previous experience [19,20] recent reports that oxidants other than O_2 are able to produce 'activated' forms of the oxidized enzyme [14]. Finally, with the use of $H_2{}^{17}O$, we have attempted to ascertain whether labile oxygen ligands (bridging O^{2-}, OH^- or H_2O) are contained within the substructure responsible for these signals.

Materials and Methods

Cytochrome *c* oxidase was purified from beef heart mitochondria using the procedure developed by Hartzell and Beinert [22,23]. A sample of en-

zyme prepared according to the method of Van Buuren (Ref. 24; and in Ref. 23, p. 61 of Ref. 14 cited therein) was a gift of Dr. S.P.J. Albracht. The buffer composition used throughout these studies was 5 mM Tricine, 5 mM cacodylate (Na^+) and 0.2% (w/v) Tween 20 at a pH of 7.2. Final enzyme concentrations (expressed in terms of total heme *a*) were generally around 0.5 mM. Samples of NADH (Boehringer Mannheim Co.), $K_3Fe(CN)_6$ (Matheson, Coleman and Bell) and porphyrexide (K & K) were used as purchased. Reduction of cytochrome *c* oxidase samples was, unless otherwise stated, carried out by anaerobically mixing enzyme with a stoichiometric amount of NADH (lyophilized solution) and allowing 12–18 h for full reduction at 4°C. Oxygen solutions were prepared by bubbling O_2 through buffer solution incubated at 17°C. Glass syringes housed within steel barrels were used in rapid-mixing experiments.

Optical spectra were obtained using a versatile rapid-scanning instrument, some features of which have been developed in this laboratory. Rapid mixing of reactant solutions was accomplished by variable-velocity syringe displacement using an electric ram system (Update Instruments Inc.). Nylon incubation hoses, three-grid lucite mixer, calibrated aging hose (also nylon) and stainless-steel observation chamber (optical path length 2.1 mm) were housed in a sealed bath through which a 1 : 1 water/ethanol mixture at 2°C was circulated. The reaction time for transient spectra obtained in the continuous-flow mode could be adjusted by variation of aging-hose length or ram velocity. In the case of stopped-flow experiments, the shortest hose and highest ram velocity were used in order to minimize the system dead time (approx. 10 ms). In order to obtain transient spectra in the 2–5 ms time range, a combination mixing-observation microchamber (total volume 6 μl) was constructed. The experimental aging time was varied by suitable selection of ram velocity.

Rapid-scanning was carried out using a Tracor Northern TN1710 spectrometer in conjunction with a TN1223 optical detector system. The spectrometer was equipped with the following modules: 1710-21, optical spectrometer; 1710-37, sequential scan; 1710-9, data processor; 1710-29, photometric processor; 1710-8, calibrator; 1710-28, memory subgroups. Absorption spectra were obtained by logarithmic comparison of incident transmission spectra with reference (buffer) spectra. For spectra in the wavelength region 400–750 nm, a high-pressure Xe arc source (Osram XB0, 150 W) was employed, whereas a tungsten lamp (108 W) was used for near-infrared studies (600–900 nm). Wavelength calibration was carried out using Xe emission lines at 467 and 687 nm, or by use of a didymium filter for tungsten lamp radiation.

For transient continuous-flow studies, acquisition of spectra (scan time 5 ms for 300 nm) was actuated by a ram-displacement-controlled trigger signal adjusted to permit adequate flushing of the system without overconsumption of solution material. Flow was terminated after the further displacement calculated to be sufficient for the sequential acquisition of eight spectra. At constant flow velocity in a continuous-flow experiment these are time independent and undistorted. Eight transmission spectra were averaged and processed in each case. Data were stored in cassettes using a TN1114 recorder in conjunction with a TN1316 interface.

For stopped-flow data, acquisition of spectra was actuated by circuit completion at a stop-syringe valve. A total of 14 spectra could be obtained with separation times varying from 10 ms to 100 s. However, a disadvantage with this mode is the less satisfactory signal-to-noise ratio due to nonaveraging of spectra, and the appearance of spectral distortion in the case of reactions that are rapid in comparison with the scan time.

Manually actuated spectra were obtained with reference to a stopwatch. The averaging procedure was as described above.

Kinetic samples for EPR analysis were obtained by rapid mixing of reactant solutions. Mixed solutions were expelled into EPR tubes and frozen in a liquid isopentane bath at -140°C following periods of incubation at 2°C. Reoxidation of stoichiometrically reduced (NADH) cytochrome *c* oxidase by porphyrexide was carried out by anaerobically mixing enzyme solution with lyophilized (from a 100 mM solution in H_2O) porphrexide at 4°C. The final concentration after dissolution was 18 mM. EPR measurements at 9.2 GHz were conducted as described previously [19,20,22].

Experiments to compare the EPR spectra of O_2-reoxidized enzyme in $H_2{}^{16}O$ and $H_2{}^{17}O$ were carried out using the rapid-mixing procedure. the sample of $H_2{}^{17}O$ (60% enrichment) was purchased from Merck, Sharp and Dohme (Canada Ltd.) and used to prepare O_2-saturated buffer and enzyme solution. Cytochrome *c* oxidase solution (1.93 mM) was further concentrated by ultracentrifugation at 192 000 × *g* for 20 h at 2°C. The top layer of water was removed and the enzyme was resuspended in a concentrated stock solution of Tricine, cacodylate, Tween 20 and $H_2{}^{17}O$. The final buffer and enzyme concentrations after rapid mixing were 5 and 0.94 mM, respectively, and the final ^{17}O enrichment in the water was calculated to be 43–46%. Control samples were prepared by the same procedure from the same concentrated enzyme solution with the exception that no $H_2{}^{17}O$ was added. The spectra from which line-width comparisons were made represented the averages of 36 scans each.

Results

Reoxidation with oxygen

Following rapid reoxidation of the stoichiometrically reduced form by oxygen, cytochrome *c* oxidase undergoes several spontaneous transformations as generally evident by reference to Fig. 1. Variations in the shape and intensity of the 655 nm band, accompanied by a gradual absorbance decrease around 600 nm, are reproducible features which have been noted in all our experiments and at least two phases can be kinetically resolved. We may discuss the results in terms of two empirical phases, A and B, recognizing that phase B certainly comprises multiple processes. Owing to the small relative magnitude of absorbance changes involved, we have made use of time-difference spectra to clarify detail. A summary of our observations is shown in Table I.

Phase A involves the disappearance of an absorbance enhancement at 660 nm, which is developed at earliest resolvable times (less than 10 ms). This process is exponential with a half-life of 46 s, and the amplitude corresponds to $\Delta\varepsilon = 0.25$ mM (heme *a*)$^{-1} \cdot$ cm^{-1}. Further optical changes associated with this phase, as determined by 1–75 s time-difference analysis, are seen in Fig. 2. The use of a small time interval minimizes contribution from phase B while allowing the generation of sufficient absorbance amplitude from phase A. Clearly, there is a well defined band at 660 nm with a trough at 580 nm. The small feature at 600 nm, which becomes more pronounced at longer times, is associated with phase B. Within 3 min phase A is complete and the oxidised enzyme

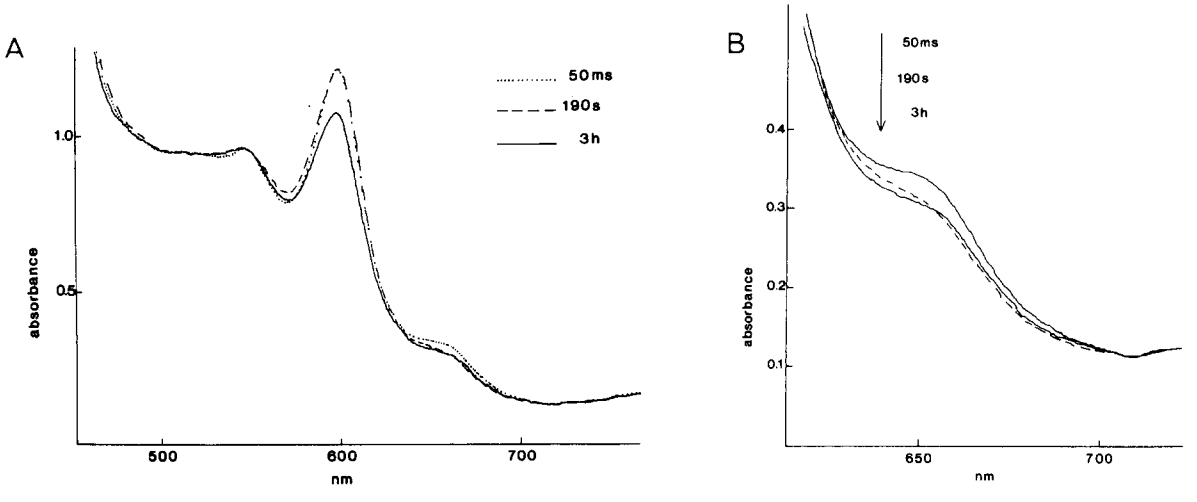

Fig. 1. (A) Absorption spectra of cytochrome *c* oxidase at various times following reoxidation of the stoichiometrically (NADH)-reduced form with O_2. Experimental conditions were: enzyme 0.5 mM (total heme *a*), O_2 0.5 mM, temperature 2°C, pH 7.2 (5 mM Tricine, 5 mM cacodylate, 0.2% (w/v) Tween 20). Optical path length 2.1 mm. (B) Enlargement showing variations in the 655 nm absorption band.

TABLE I

SUMMARY OF TIME-DEPENDENT VARIATIONS IN THE VISIBLE AND EPR SPECTRA OF CYTOCHROME *c* OXIDASE FOLLOWING RAPID REOXIDATION OF REDUCED ENZYME BY O_2 AT 2°C

	Visible	EPR
Phase A	Band at 660 nm (formed in less than 10 ms) decays with $t_{1/2}$ (2°C) approx. 46 s, $\Delta\varepsilon = 0.25$ mM (heme a)$^{-1}\cdot$cm^{-1}. A simultaneous increase in absorbance occurs at 580 nm.	Resonances at g 1.78 and 1.69 shift downfield by 16–20 G, accompanied by some broadening at g 1.78. No observable change at g 5.
Phase B	Slow decrease in intensity of the α-band ($t_{1/2}$ approx. 40 min at 2°C) is accompanied by loss and gain of small features at 640 and 665 nm, respectively. Complex time dependence indicates involvement of several species.	Resonances at g 5, 1.78 and 1.69 decrease in intensity. Time course parallels the decrease in α-band intensity.

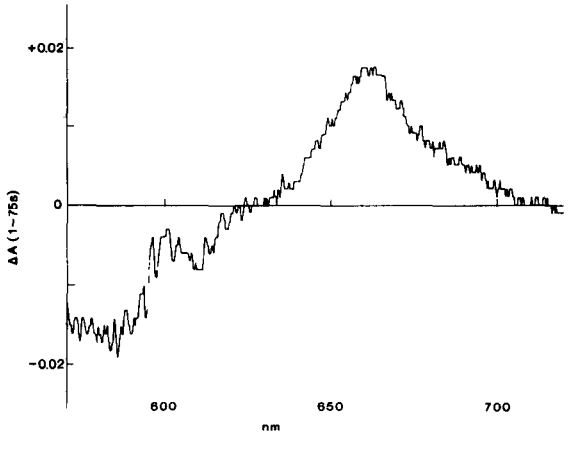

Fig. 2. Time-difference spectrum (1–75 s) following reoxidation of stoichiometrically (NADH)-reduced cytochrome *c* oxidase by O_2. Experimental conditions were the same as described for Fig. 1.

exhibits a rather obtuse absorption at 655 nm.

Phase B comprises the processes that continue to occur over a time span of 3 h, at which point the visible spectrum of the resting enzyme is virtually restored. The time-difference spectra in Fig. 3 show the nature of the spectral changes involved. The decrease in absorbance at 600 nm is accompanied by loss and gain of small features at 640 and 665 nm, respectively, which together serve to restore sharpness to the 655 nm band. Involvement of other processes is indicated by changes in the shape of time-difference spectra at longer times, and this is substantiated by kinetic analysis employing the Guggenheim approximation (Fig. 4). Using data for 597 nm, a plot of $\log(A_{t1} - A_{t2})$ vs. time ($t_2 = t_1 + 6000$ s) is linear to the extent of only one half-life (40 min). Analysis of early time points (less than 200 s) indicates that there is a slight delay in the absorbance decrease at 597 nm; however, the small changes involved do not permit distinction between the initial increase (below 630 nm) incurred during phase A (Fig. 2) and possible consecutive behavior.

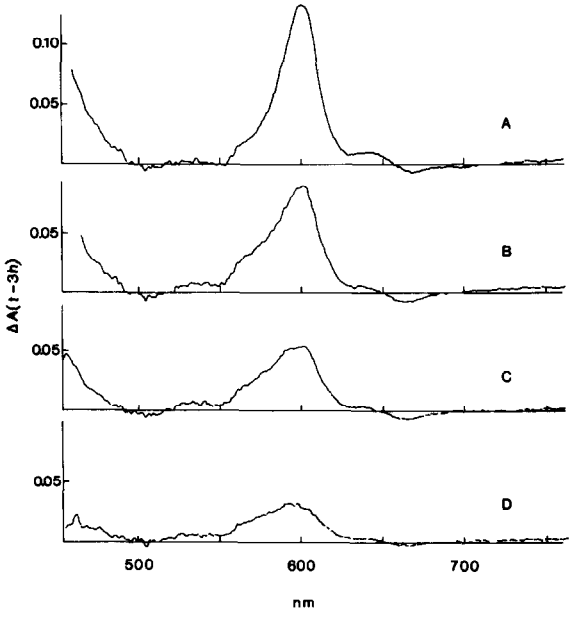

Fig. 3. Time-difference spectra ($t - 3$ h) following reoxidation of stoichiometrically reduced cytochrome *c* oxidase by O_2. Experimental conditions were the same as described for Fig. 1. (A) 195 s − 3 h, (B) 25 min − 3 h, (C) 50 min − 3 h, (D) 90 min − 3 h.

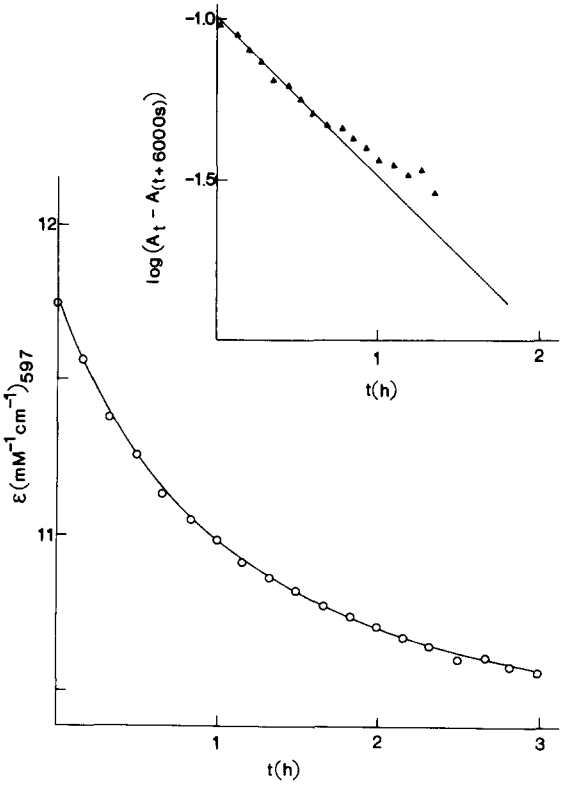

Fig. 4. Profile for decrease in absorbance (597 nm) following reoxidation of stoichiometrically reduced cytochrome *c* oxidase by O_2. Inset shows Guggenheim plot ($\log(A_t - A_{(t+6000\,s)})$ vs. time). Experimental conditions were the same as described for Fig. 1.

Stopped-flow experiments to investigate the time range 10 ms to 1 s, as well as attempts to detect earlier species (less than 5 ms) using the microchamber, yielded no meaningful evidence for other processes. The earliest spectra (approx. 2 ms) indicated the presence of small amounts of reduced enzyme but predominantly supported the conclusion that formation of the enhanced 660 nm absorption is very rapid. Studies directed at monitoring changes in the broad 830 nm band were also carried out, and poorly defined variations in height and width were observed; but these did not interfere with the spectrum below 700 nm.

Because of the rather exacting requirements for stoichiometric prereduction of enzyme, control experiments to examine possible artifacts resulting from excess or insufficient NADH were performed. With an 8-fold excess of NADH no difference was detectable with regard to phase A, but slow rereduction became apparent at longer times. In the case of incomplete (90%) prereduction, the initial enhancement at 660 nm was less pronounced while the early stages of phase B (including the region overlapping with phase A) were masked by a more rapid absorbance decrease at 600 nm, possibly attributable to reoxidation of components not in effective electron-transfer communication with O_2. The presence of trace amounts of TMPD or cytochrome *c* (less than 1% of total heme *a*) did not affect absorption spectra or kinetic behavior.

At the earliest sample time obtainable from manual-freezing experiments (same solution conditions as for the optical study), which is nominally 1.5 s, not including freezing time, the EPR resonances attributable to low-spin heme and EPR-detectable Cu are fully formed. Variations in the shape of the heme signals over a 3 h period at 2°C did not follow any meaningful pattern. On the

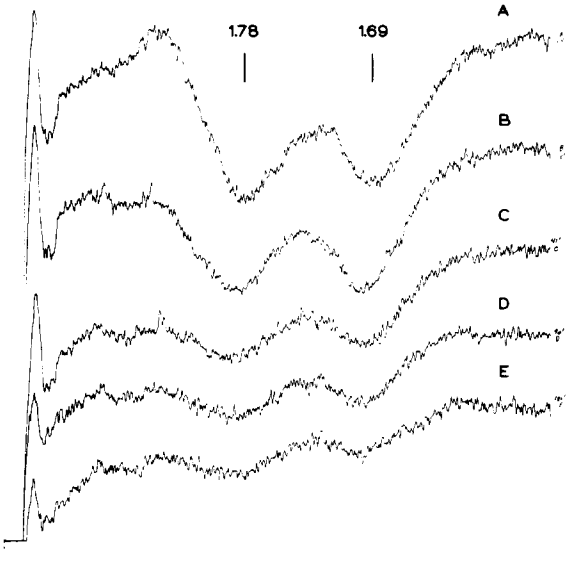

Fig. 5. EPR signals *g* 1.78 and *g* 1.69 at various times following reoxidation of stoichiometrically reduced cytochrome *c* oxidase by O_2. Experimental conditions were the same as described for Fig. 1. Conditions for EPR spectra were: microwave power 10 mW, frequency 9.22 GHz, modulation amplitude 8 G, modulation frequency 100 kHz, temperature 13 K. (A) 1.5 s, (B) 5 min, (C) 41 min, (D) 90 min, (E) 3 h. All spectra represent the average of nine scans.

other hand, changes in the shape, position, and size of the 'novel' resonances at g 5, 1.78 and 1.69 (Fig. 5) correlate rather well with the optical profiles described above. While there is little change at g 5 during the interval required to complete phase A, the resonances at g 1.78 and 1.69 shift downfield by 16–20 G and the g 1.78 component broadens. The time dependence of the 'g 1.78' field shift, as shown in Fig. 6, closely matches the decrease in absorbance at 660 nm. Following this, resonances at g 5, 1.78 and 1.69 (we retain this nomenclature despite the small field shift) decrease in intensity. The difficulties involved in defining a suitable baseline prohibit the accurate measurement of signal intensity necessary for a rigorous kinetic treatment. Nevertheless, as shown in Fig. 7, estimates of the height of the g 1.78 resonance provide a reasonable correlation with the α-band absorbance decrease measured at 597 nm. Appearance of a g' approx. 12 resonance at longer times may also be relevant, perhaps in regard to the additional optical complexities apparent in phase B.

Support for these correlations stems from a parallel examination using cytochrome c oxidase prepared according to the procedure of Van Buuren (Ref. 24; and in Ref. 23, p. 61 of Ref. 14 cited therein). The sample was considerably autoreducible as evident from the occurrence of extensive reduction merely upon anaerobization. The initial enhancement at 660 nm was of lower amplitude and the optical and EPR variations assigned above to phase A were both retarded. The half-life for 660 nm decay was 15–20 min and the shift in g values was similarly affected. The decrease in absorbance at 600 nm defining phase B occurred at a comparable rate to that measured with the sampled prepared according to the method of Hartzell and Beinert [22,23] but appeared to terminate prematurely. The same profile of changes was observed for the g 5, 1.78 and 1.69 EPR resonances. There was little further change between 1 and 3 h.

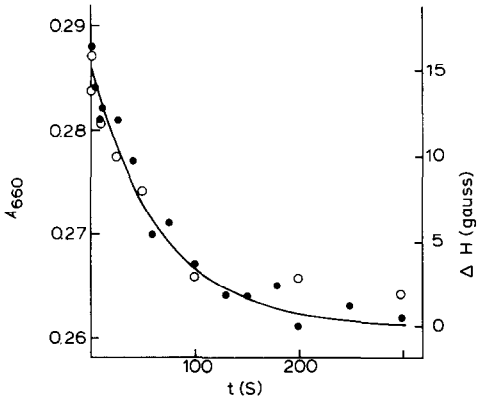

Fig. 6. Comparison of the time dependence of the field shift observed for the g 1.78 signal and the decrease in absorbance at 660 nm. Experimental conditions were the same as described for Fig. 1. Conditions for obtaining the required EPR spectra were the same as described for Fig. 5. (●) Absorbance decrease at 660 nm, (○) field shift.

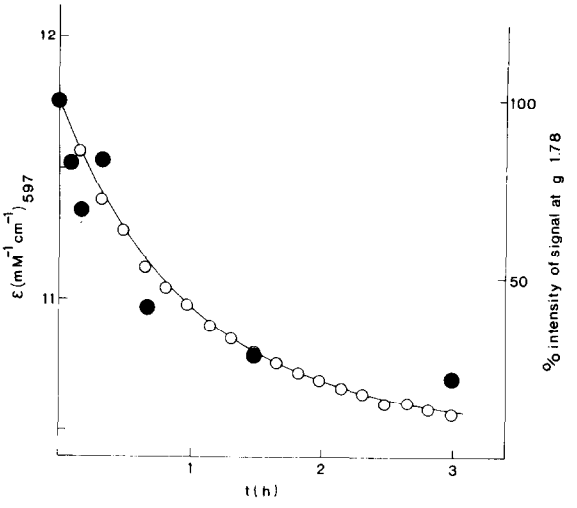

Fig. 7. Comparison of the profiles for absorbance decrease at 597 nm (○) and variation in the intensity of EPR signal at g 1.78 (●) following reoxidation of stoichiometrically reduced cytochrome c oxidase by O_2. Experimental conditions were as described for Figs. 1 and 5.

Reoxidation with ferricyanide and porphyrexide

Some previous studies in this laboratory have been concerned with the early oxidized forms of cytochrome c oxidase produced by anaerobic reoxidation. Such forms were characterized by the lack of a prominent absorption at 655 nm along with the generation of an intense rhombic EPR resonance around g 6, attributable to high-spin cytochrome a_3. In this study actual 'reoxidation' by $K_3Fe(CN)_6$ (5 mM $K_3Fe(CN)_6$, 0.5 mM heme a), as judged by the decrease in intensity of the reduced form α-absorption, is slow; and develop-

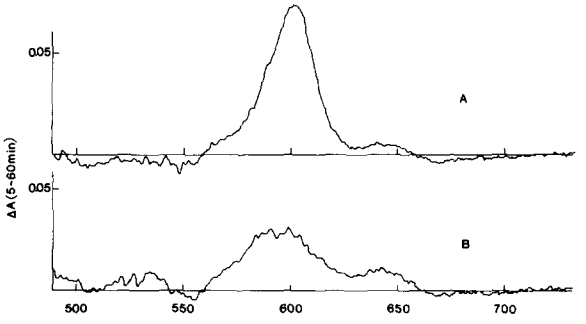

Fig. 8. Time-difference spectra (5–60 min) for cytochrome *c* oxidase reoxidized using: (A) O_2 (0.5 mM), (B) $K_3Fe(CN)_6$ (5 mM). The conditions of enzyme concentration, temperature and pH were as described for Fig. 1.

ment of the 655 nm band is seen to occur over a period of 0–60 s to approach a form similar to that obtained at the end of phase A in aerobic oxidation. After these initial processes, however, the time-difference spectrum obtained (5–60 min) bears a close resemblance to that of the O_2-reoxidized enzyme (Fig. 8). The kinetics are somewhat different, however, more of phase B occurring within 5 min than with the aerobic form.

Following this it was naturally decided to ascertain whether the EPR resonances associated with aerobic reoxidation could be generated anaerobically. For this purpose porphyrexide was chosen as oxidant in order to obtain less interference in the EPR spectrum than is possible with $Fe(CN)_6^{3-}$. For two titration-type samples, frozen at 6 and 23 min, the required resonances were obtained in almost the expected amount as judged from relative time-difference optical comparison with Fig. 8. The 6 min spectrum is shown in Fig. 9b along with a 5 min O_2-reoxidized sample (Fig. 9a).

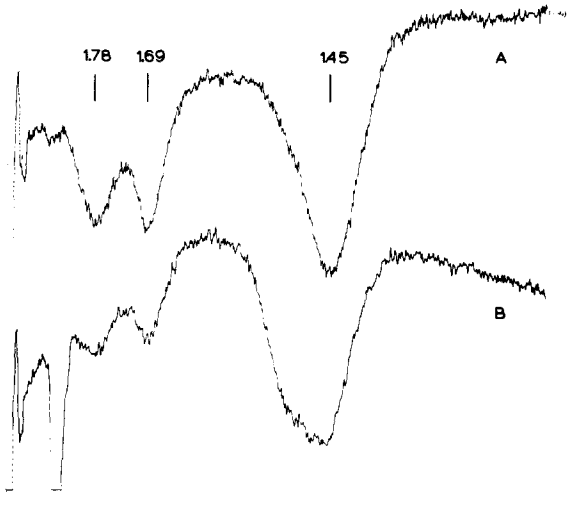

Fig. 9. EPR spectra of cytochrome *c* oxidase: (A) 5 min after reoxidation with O_2, (B) 6 min after reoxidation with porphyrexide. Conditions for obtaining EPR spectra were as described for Fig. 5.

Effect of $H_2{}^{17}O$ on EPR spectrum

Comparison of the *g* 1.78 and 1.69 resonances for similar enzyme samples mixed in O_2-saturated $H_2{}^{16}O$ and $H_2{}^{17}O$ (43–46% final enrichment) yielded no evidence for isotopic superhyperfine broadening from the ^{17}O nucleus. For example, the average line widths of the *g* 1.78 resonance at half-minimal amplitude of the $H_2{}^{16}O$ and $H_2{}^{17}O$ samples were 83 ± 1 and 84 ± 2 G ($\pm$S.E.; $n = 2$) respectively.

Discussion

For discussion of our results we have adopted an empirical approach, chronologically assessing possible relationships with transient EPR signals, and comparing our results with various observations reported by other investigators. In this respect it seems most convenient to begin with phase B, the dominant visible feature of which is a slow, approx. 10–15% decrease in α-band intensity accompanied by an approx. 3 nm shift to shorter wavelength as indicated by Figs. 1 and 3. Despite many differences in experimental conditions, particularly in the manner of reduction and reoxidation, it seems very likely that the slow processes that we observe are essentially analogous (but see later) to those reported by other investigators [2,8].

It is difficult, however, to assess the relationship of phase A, as observed in our experiments, with previous observations that have been reported in the literature. Indeed, a clearcut distinction between phases A and B was discernable only with use of cytochrome *c* oxidase samples prepared according to the procedure of Hartzell and Beinert. With the sample prepared according to Van Buuren, absorbance changes associated with phase A were somewhat milder in terms of amplitude and certainly much slower, although these dif-

ferences could be related to the autoreducibility of this particular sample. Therefore, it is quite likely that phase A could go undetected in other studies, particularly in experiments involving a significant time delay between 'O_2-pulsing' and spectral measurement. As far as we are aware, the dominant feature of phase A, the loss of a prominent absorption component at 660 nm which serves to decrease markedly the initial sharpness of the 655 nm shoulder has not been previously reported. The shoulder at 655 nm has been postulated as representing a form of the enzyme in which Fe(III) cytochrome a_3 and 'EPR-undetectable Cu(II)' are in a spin-coupled state [15] and it is likely that the extent of interaction between the two centers is being modulated, possibly by conformational transitions. Visible changes at about 640–660 nm, that eventually restore sharpness to the 655 nm shoulder, follow in the course of phase B. Again, we are not aware of independent observations that might be relevant to this discussion. As a result of the absorbance increase at 580 nm that accompanies the decrease at 660 nm (Fig. 2), spectra recorded a few minutes after reoxidation with O_2 not only show a less pronounced 655 nm shoulder but also display a higher 'trough' absorbance level at about 580 nm. Consequently, as shown in Fig. 1, the level of the 580 nm trough in the 50 ms spectrum is similar to that observed after 4 h, whereas at intermediate times, i.e., 190 s, it is actually somewhat higher. Comparison with other published spectra reveals two examples from which a similar situation can be inferred. The spontaneous decay of the so-called 'Compound I' to give 'Compound II' as defined by Orii and King [8] occurs with a half-life of 3 min at 22°C and appears (in part) to involve a small increase at 578 nm. Spectral details were not examined at wavelengths greater than 620 nm. Of note, however, is the significant decrease at 605 nm observed by these authors during this stage. As apparent from our results, spectral changes associated with phase A alone actually give a small but measurable increase in α-band intensity prior to the large decrease in phase B. Presumably the delay in spectral scanning, 1 min as reported by the authors, could have concealed this feature. The second example in which a relatively low absorbance level at 580 nm is apparent is the 'pulsed' form of Brunori et al. [12]. The authors analyzed stopped-flow absorbance profiles following the rapid reoxidation of the fully reduced form by O_2. Combination of data corrected to 'zero-time', obtained at various wavelengths (550–625 nm) generated the spectrum of pulsed cytochrome *c* oxidase, shown, with that of the resting enzyme for comparison purposes, in Fig. 2 of Ref. 12. While the pulsed form displays an enhanced α-band absorption, there is a slight trough (relative to that of the resting form) around 580 nm. Other published spectra [1–5] show higher absorbance levels around 580 nm and consequently, by our terms of reference, relate to species that have already undergone phase A.

Comparison of the early absorbance decrease at 660 nm with the small field shift measured at *g* 1.78 yields a legitimate correlation although no corresponding changes could be detected at the *g* 5 component. The general correlation between phase B optical changes and the decrease in size of the EPR resonances at *g* 5, 1.78 and 1.69 is also encouraging, as demonstrated in Fig. 7. However, the limited linearity of the kinetic plot (Fig. 4) and minor shape changes in the time-difference absorption spectra of Fig. 3 indicate that additional processes are operative. It is possible that the small variations at longer times may be related to formation of the unusual EPR signal at *g'* approx. 12, which we observe in agreement with Brudvig et al. [21]. Previously, we reported that the *g* 5, 1.78 and 1.69 resonances decayed with a half-life of the order of 100 s at 16°C. Our original concept of the species giving rise to this EPR spectrum, namely that it is a transient form of the oxidized enzyme, clearly requires further qualification. From our latest experiments it is clear that at least two forms of the oxidized enzyme, displaying similar EPR spectra, appear and subsequently decay following rapid reoxidation by O_2. The species disappearing in phase B, for which we can use the terminology '*g* 5" to distinguish it from the earlier species having the more intense 655 nm shoulder (the *g* 5 form), can be considerably stabilized at temperatures near 0°C. This helps to rationalize previous observations of small signals at *g* 1.785 and 1.694 in resting samples [22]. At the present time, uncertainty regarding the exact spin multiplicity of the system responsible for these signals precludes attempts to determine the fraction of metal compo-

nents involved. The unusual temperature dependence, maximum intensity at 13 K [19], provides an additional complication in quantitative determinations.

As illustrated in Fig. 8, difference spectrum features (5–60 min) generated following anaerobic reoxidation by $Fe(CN)_6^{3-}$ are very similar to those seen in O_2-reoxidation experiments. On the basis of the relationship between optical and EPR spectra as discussed above, it is no surprise that EPR signals at g 1.78 and 1.69, as expected for the g 5′ form, are observed in anaerobically reoxidized samples frozen after 6 min. This result is significant in the light of a recent report concerning the formation of the so-called pulsed oxidase by anaerobic reoxidation with $Fe(CN)_6^{3-}$ [14]. Previous studies in this laboratory have focused upon the form identified by the presence of intense rhombic EPR signals around g 6 and the absence of a well developed shoulder at 655 nm, that is produced within several milliseconds by rapid mixing of reduced oxidase with anaerobic solutions of $Fe(CN)_6^{3-}$ or porphyrexide [15]. After a 6 min incubation period with porphyrexide we find that the rhombic high-spin signals are greatly decreased in intensity.

The question of whether the species exhibiting EPR resonances at g 5, 1.78 and 1.69 retains, in any form, products of O_2 reduction has been considered in two previous contributions from this laboratory. No isotopic superhyperfine broadening effects at g 1.78 and 1.69 could be seen following reoxidation by 90% enriched $^{17}O_2$ [20], while mass spectrometric analysis of water produced during single-turnover reoxidations with $^{18}O_2$ failed to provide any evidence for O-atom retention [25]. The finding that reoxidation of the enzyme in approx. 45% enriched $H_2{}^{17}O$ produced no discernible line broadening now provides some evidence against the presence of a labile O-ligand ($\mu \cdot O^{2-}$, OH^-, H_2O) in the g 5 species. It is our view that a 10% broadening effect, i.e., approx. 4 G, in either direction from the 80 G wide line, would be easily detected.

The relevance of these alternative forms in the catalytic function of cytochrome c oxidase remains unclear. Recent experiments have indicated that intramolecular electron transfer between cytochrome a and one of the Cu atoms, and the component(s) giving rise to the signals at g 5, 1.78 and 1.69, might be too slow to be compatible with high turnover rates observed in steady-state kinetic studies (Shaw, R.W., Beinert, H. and Armstrong, F.A., unpublished results). However, assumptions made that (a) the novel signals do arise from the site of O_2 reduction and represent a significant proportion of the total cytochrome a_3 or copper content, and (b) high levels of aggregation, likely to be considerable in concentrated enzyme solutions, do not greatly affect activity, require closer scrutiny. Steady-state kinetic measurements, using enzyme solutions of varying detergent composition, have indeed served to emphasize the role played by solubilizing agents in modifying the activity of cytochrome c oxidase [26]. Consequently, we feel that some caution is required in any evaluation of the catalytic and physiological importance of these species.

Acknowledgements

We are indebted to Mr. R.E. Hansen for his valuable collaboration. This work was supported by a research grant (GM-12394), a Research Career Award (5-K06-GM-18492) to H.B. and a National Research Service Award (S-F32GM-05772) to R.W.S. from the Institute of General Medical Sciences, National Institutes of Health, U.S.P.H.S. We are grateful to Professor R.J.P. Williams and Dr. H.A.O. Hill for helpful comments during the preparation of this manuscript.

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