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Electron Carriers of Cytochrome Oxidase Detectable by Electron Paramagnetic Resonance and Their Relationship to Those Traditionally Recognized in This Enzyme

(cytochrome *a*, a_3/low-temperature spectra/rapid kinetics/redox titrations)

CHARLES R. HARTZELL*, RAYMOND E. HANSEN†, AND HELMUT BEINERT†

* Department of Biochemistry, Pennsylvania State University, University Park, Pa. 16802; and † Institute for Enzyme Research, University of Wisconsin, Madison, Wis. 53706

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ABSTRACT On the basis of oxidoreductive rapid kinetic and titration experiments with purified cytochrome *c* oxidase (EC 1.9.3.1), monitored by electron paramagnetic resonance (EPR) at 13°K and by spectrophotometry at 100°K, a new assignment of EPR signals is proposed. The bulk of both the low-spin (g = 3.0; 2.2; 1.5) and high-spin (g = 6; 2) signals is attributed to the component with the properties of traditional cytochrome *a*. It is further proposed that the absorption band at 655 nm represents the most unambiguous manifestation of the a_3 component.

With the advent of new tools of investigation the enzyme cytochrome *c* oxidase (EC 1.9.3.1) has received considerable attention. Low-temperature electron paramagnetic resonance (EPR) spectroscopy has made it possible to detect copper and several heme species in the enzyme depending on the oxidation state. On the basis of EPR observation at 90°K Van Gelder and Beinert (1) proposed a tentative assignment of the detectable signals, but during EPR spectroscopy at lower temperatures it became apparent that the simple interpretation of the spectra suggested by these authors was not able to account for observations made by EPR at about 10°K (1, 2) and by other approaches (3–8). Particularly, information obtained from measurements of redox potentials (3–8) was not compatible with the proposal that the bulk of the EPR signal of a high-spin heme component, which appears at intermediate states of oxidation, represents the a_3 component, which is defined as the species that, in its reduced state, reacts with O_2 or CO and is thought to have a high redox potential. It was therefore proposed (4) that a low-spin heme component (g = 3.0; 2.2; 1.5), which is detectable in the oxidized form of the enzyme and the signal of which disappears with the first equivalents of reductant added, represents the high-potential heme of the enzyme and thus presumably a_3, whereas the signal at g = 6 was attributed to *a*.

From a large number of titrations and rapid kinetic experiments, involving different reductants and oxidants and monitored by low-temperature EPR (13°K) and optical reflectance spectroscopy (100°K), we would like to propose here an interpretation, which differs from those brought forth thus far, but which, at this time, appears to us to be most compatible with the results obtained by equilibrium and rapid reaction approaches as well as with the older spectrophotometric results, which have led to the concept that two substantially different cytochromes, *a* and a_3, are present.

The principal difficulty, too often ignored in interpretations of the EPR spectra of cytochrome *c* oxidase, is the determination of the quantitative relationships between the components represented in the detectable signals. Of the total copper or heme present in the oxidized form of the enzyme only 30–40% can be detected by EPR. The behavior of the remaining components must be inferred from indirect evidence or optical data, which in themselves are not interpretable in exact quantitative terms.

Ferrous heme and cuprous copper produce no EPR signals. Therefore, appearance or disappearance of signals could be and has been equated with oxidation or reduction, respectively, of the corresponding components. Since, however, at no time more than half of the potential electron carriers are detectable, such an interpretation is not unambiguous; disappearance into the undetectable pool or replenishment from this pool might also occur. Furthermore, since the shape of the heme signals depends on various factors (see *Discussion*), even comparisons of related signals based on signal size are fallacious.

The apparent discrepancies in the information available on *c* oxidase, which lead us to the present proposal, are the following: in titrations the low-spin heme signal at g = 3 disappears immediately with the first electrons entering the enzyme. This has led to the interpretation that disappearance indicates reduction of that component (1, 4) and that the g = 3 signal represents the component of highest redox potential and thus presumably a_3 (4). In rapid-reaction experiments, however, with *c* or dithionite as reductant, it is also the signal at g = 3 that disappears immediately. If the above assignment of signals were correct, and disappearance of signal is again equated with reduction, this would mean that a_3 is rapidly reduced, which contradicts conclusions drawn from spectrophotometric studies, namely that reduction of a_3 is slow (9–12).

Furthermore, the ligand-binding properties of what has generally been considered to be a_3 from spectrophotometric work, are those expected of a high-spin rather than a low-spin heme compound, whereas the signal at g = 3 is clearly that of a low-spin heme.

We are aware that a number of these arguments are based on interpretations of spectrophotometric data, not on unam-

Abbreviations: EPR, electron paramagnetic resonance; *a*, a_3, *c*, cytochrome *a*, a_3, or *c*.

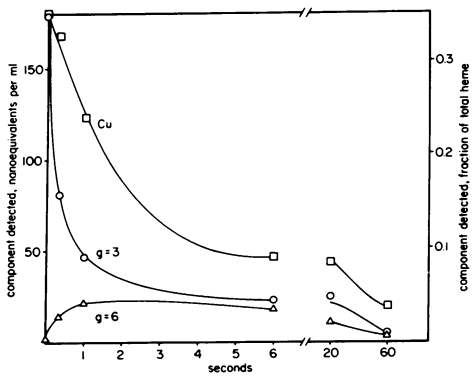

FIG. 1. Time-course of anaerobic reduction of 517 μM *c* oxidase by 7 mM dithionite (dissolved in 0.05 M Tris base). The *abscissa* indicates time in seconds, the *ordinate* the absolute concentration (*left*) or concentration relative to the spectrophotometrically determined heme concentration (*right*) of the detected components according to integration of the signals (see *Methods*). Symbols: ○, low-spin heme ($g = 3$); □, copper; △, total high-spin heme ($g = 6$).

biguous facts. Nevertheless, as a whole, they cannot be lightly ignored. There is one piece of experimental evidence that strongly supports the argument that a_3 is indeed slowly reduced, namely the delayed appearance of photosensitivity when *c* oxidase is reduced by *c* in the presence of CO (9). If a_3^{2+} is the component combining with CO—a point on which there seems to be general agreement—and if the rate of combination of a_3^{2+} is as stated (9, 13, 14), then this is in contradiction to the notion that the disappearance of the EPR signal at $g = 3$ indicates reduction of a_3^{3+}.

The following interpretation removes this contradiction: the low-spin heme signal at $g = 3$ represents largely, if not exclusively, *a*. This finding agrees with the kinetic results (9–12) and the observation of delayed emergence of photosensitivity on reduction in the presence of CO (9). This interpretation can also be brought into agreement with the information on redox potentials (3–8) *if it is considered that absence of the $g = 3$ signal does not necessarily indicate that this component is reduced.* We propose that on the time scale in-

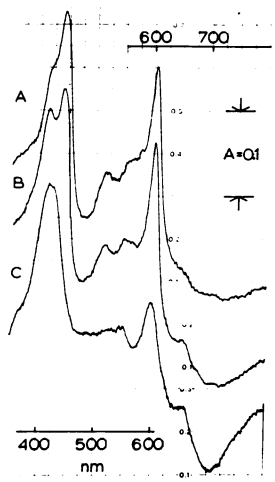

FIG. 2. Low-temperature reflectance spectra of the experiment of Fig. 1. The spectra shown refer to the samples frozen after 1 min (*A*) and 20 sec (*B*), and to the oxidized control (*C*).

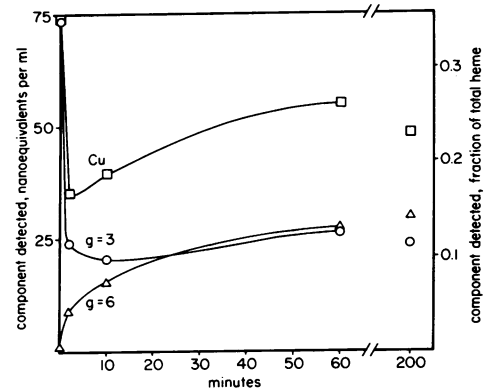

FIG. 3. Time-course of reduction of 0.23 mM *c* oxidase by 0.12 mM dithionite. The anaerobic titration technique with solid dithionite (25) was used. The *abscissa* shows time in minutes. The *ordinates* and *symbols* are as in Fig. 1.

volved in titration experiments (minutes), *a*, although initially reduced according to the evidence from kinetics, is reoxidized by enzyme components of higher redox potential and reappears in a high-spin ferric form with a signal at $g = 6$. Thus, according to this interpretation, the bulk of both detectable heme signals ($g = 3$ and $g = 6$) of *c* oxidase is due to the *a* component. This is in agreement with the experimental observations that (*1*) as the $g = 3$ signal disappears during reductive titrations (time scale, minutes) one or more components of high potential are reduced and (*2*) that the component represented in the $g = 6$ signal is of low redox potential (4). On the time scale of rapid kinetic experiments (milliseconds), the signal at $g = 6$ is generally found to be smaller than in titration experiments, indicating, in terms of our interpretation, that in these instances *a* is indeed largely present as a^{2+} and is not yet reoxidized by the component(s) of higher potential. This again is in agreement with previous spectrophotometric observations, namely that with purified *c* oxidase the reduction of a_3^{3+} by a^{2+} is very slow.

Since we have observed not a single but a great number of subspecies of the signals at $g = 3$ and $g = 6$‡, we consider it possible that under specific conditions one or more of these may represent a_3^{3+}. However, we think that in attempts to sort out the main events observable with this enzyme, as a first approximation, *a_3 may be considered undetectable by EPR.*

The question, therefore, arose: are there any unique and direct manifestations of the a_3 component? We do not consider the light absorptions in the region of 445 and 605 nm sufficiently discriminating to qualify. However, according to our observations, the weak absorption band centered at 655 nm shows the behavior expected of the oxidized form of the high-potential heme component of the enzyme, when related to the behavior of the other components. Thus, e.g., on the time scale of titration experiments, when the $g = 3$ signal has disappeared and strong $g = 6$ signals are seen, the intensity of the 655-nm band is decreased, whereas at early times in kinetic experiments, when the $g = 3$ signal has also largely disappeared, but without appearance of a substantial signal at $g = 6$, the 655-nm band is not significantly changed. We have also observed, in agreement with results obtained by others (15), that at low temperature reduced *a* has a split Soret band (442 and 447 nm), whereas a_3 has only one Soret band at 445 nm. Thus, when the $g = 3$ signal has disappeared at early times, the split Soret band is observed, whereas at later times,

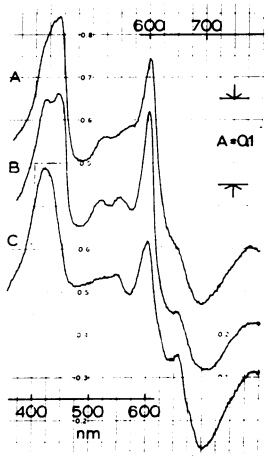

FIG. 4. Reflectance spectra of the experiment of Fig. 3. The spectra shown refer to the samples frozen at 200 min in the presence (*A*) and absence (*B*) of carbon monoxide and the oxidized control (*C*).

when the 655-nm band has decreased, absorption at 445 nm rises and obscures the separation of the Soret bands of *a* (see Fig. 5 below).

METHODS

The enzyme was prepared by an unpublished procedure of C. R. Hartzell. This procedure involves extraction with Triton and solubilization with cholate. The preparation has 12–14 nmol of heme per mg of protein and a copper-to-heme ratio of ≤ 1.1. The protein is dissolved in 0.1 M potassium phosphate of pH 7.4 containing 0.5% Tween 20. All enzyme concentrations are given in terms of molarity of total heme, not distinguishing between *a* and a_3. For experiments involving rapid mixing the values for concentrations refer to the state after mixing. Anaerobic titrations, rapid kinetic experiments (16, 17), and reflectance spectroscopy at low temperature (18) were performed and evaluated essentially as described. The temperature during reaction was 16–17°C. Improvements were introduced that allow rapid mixing and freezing at very low oxygen concentrations.

EPR spectroscopy and control of temperature were as described (19, 20). The temperature was maintained at 13.30 ± 0.15°K except for recording of Cu signals, when it was 80° or 35°K. The conditions of EPR spectroscopy were: modulation amplitude, 8 G; scanning rate, 200 G per min; time constant, 0.5 sec; 3 and 0.3 mW power for heme and copper spectra, respectively. Copper signals were integrated at low power and 80°K to avoid interference by heme. The heme signals were quantitatively evaluated by partial integration of the $g = 3$ (by an unpublished method of Dr. Tore Vänngård, whose advice we gratefully acknowledge) or the $g = 6$ peaks, the latter as described (1) with ferrimyoglobin as standard. A series of integrations was performed between 6 and 60°K. Good agreement of the values was obtained over this range of temperature, indicating that the zero field splitting of the high spin component(s) of *c* oxidase is not sufficiently different from that of ferrimyoglobin (see ref. 21) to introduce a substantial error. In view of the high value of D for ferrimyoglobin, it was not necessary to measure at lower temperatures to ascertain this. The finding that under most favorable conditions (e.g., reoxidation with ferricyanide) the integrations of the $g = 6$ signal indicated that an equivalent of 30–35% of the total heme

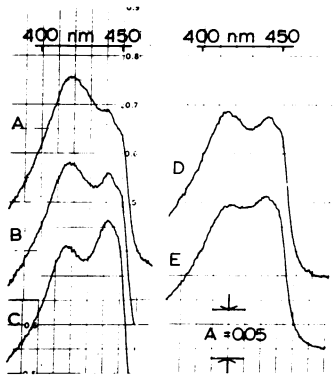

FIG. 5. Expanded reflectance spectra of Soret region of samples of experiments of Figs. 1 and 2. The spectra shown refer to the samples frozen at 340 msec (*A*), 1 sec (*B*), and 20 sec (*C*) (see Fig. 1), and at 2 min (*D*) and 200 min (*E*) (see Fig. 3).

was in this form gives us additional assurance that our quantitative estimates of this species are not grossly incorrect. In general, the $g = 6$ signal represented less heme, usually in the range of 5–15% of the total present. When *c* or ferricyanide were used, quantitative estimates of the main low-spin form ($g = 3; 2.2; 1.5$) were made by recording the high-field line at $g = 1.5$, which is not interfered with by lines from those substances. With samples less than 1 mM in heme, 4–9 signals were averaged by a Varian C-1024 or a Nicolet 1020A computer.

RESULTS

Figs. 1 and 3 show the changes in the signals of the EPR-detectable electron carriers as a function of time after addition of sodium dithionite in a rapid kinetic experiment and in a titration-type experiment, respectively. The findings agree in general with similar experiments published (1, 17). Both the low-spin heme ($g = 3$) and copper signals disappear relatively slowly with a concomitant appearance and subsequent disappearance of the high-spin signals at $g = 6$ (Fig. 1). In the

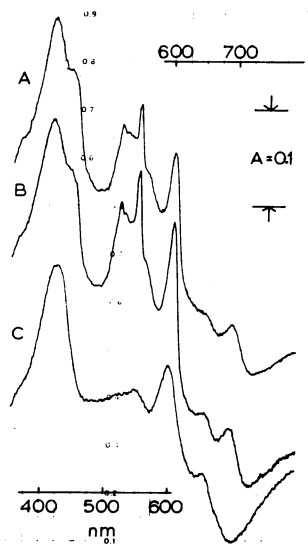

FIG. 6. Reflectance spectra of samples showing reduction of *c* oxidase by *c*. Enzyme, 1.13 mM, was mixed with 2.3 mM reduced *c* under anaerobic conditions. The spectra shown refer to samples frozen at 1 min (*A*) and 100 msec (*B*), and the oxidized control (*C*). The peak at 700 nm originates from oxidized *c*.

titration experiment (Fig. 3) the low-spin heme signal also disappears with the first reducing equivalents added. Relatively more high-spin heme is detected. The copper signal disappears initially but is apparently restored by electron redistribution within the enzyme. This has been observed consistently also with other reductants, and appears to be the reason for the observation that in titrations the copper signal does, if anything, increase rather than decrease with the first 0.5–1 reducing equivalent added per heme (1). Figs. 2 and 4 show reflectance spectra of samples of Figs. 1 and 3. It should be pointed out that in this kind of spectroscopy absorptions at longer wavelengths are emphasized, as can be seen from the strong 800- and 655-nm bands. Reflectance spectroscopy is therefore well suited for observing the behavior of the 655-nm absorption, which in room-temperature spectrophotometry is badly interfered with by changes in the slope of the bands around 600 nm. It can be seen that on the time-scale of the kinetic experiment with 14 electron equivalents of reductant per heme there is still substantial absorption left at 655 nm, even at 20 sec. Similarly, in the titration experiment with 1 electron per heme the 655-nm band disappears only slowly within hours. The presence of CO accelerates this process (Fig. 4*A*). Fig. 5 shows an expanded scan of the Soret region for some of the samples. It can be seen that the appearance of the split Soret band (442 and 447) coincides with the reduction of *a* ($g = 3$), whereas the "filling in" of the band at 445 nm parallels the slow disappearance of the 655-nm band. Results similar to those of Figs. 1–4 were obtained with *c* as the reductant, as can be seen in Fig. 6. These results agree with those of Gibson *et al.* (9) and of Andréasson *et al.* (10),

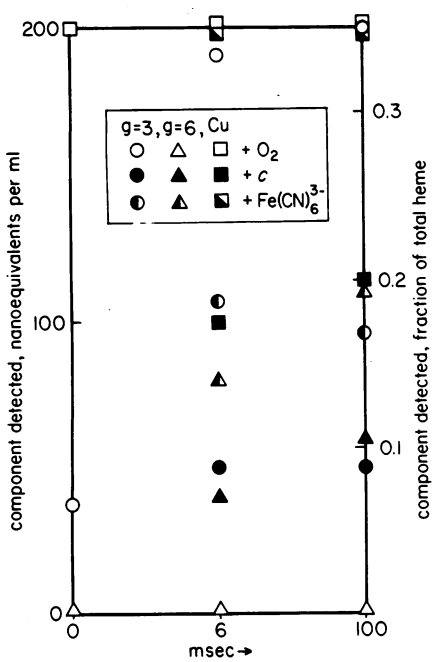

FIG. 7. Reoxidation of 575 μM *c* oxidase. 1.15 mM enzyme was reduced anaerobically by 4 mM DPNH overnight (3). Aliquots were then rapidly mixed with an equal volume of buffer saturated with oxygen, or containing 2.3 mM cytochrome *c*, or 4.6 mM potassium ferricyanide. *Abscissa* and *ordinate* are analogous to those of Fig. 1. The code for the symbols is shown in the Figure. The zero-time values (reduced sample) were identical for all samples and are therefore marked by only one type of symbol.

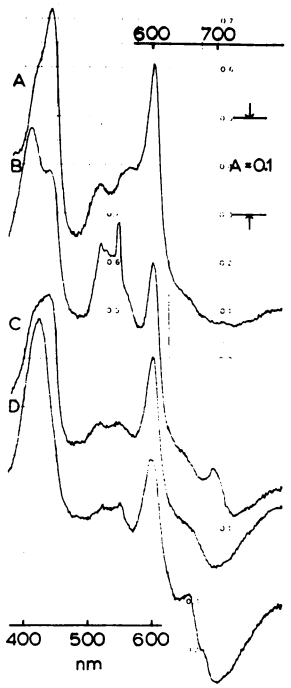

FIG. 8. Reflectance spectra of the experiment of Fig. 7. The spectra shown refer to the samples frozen at 100 msec after addition of cytochrome *c* (*B*) or ferricyanide (*C*), at 6 msec after addition of oxygen (*D*), and to the reduced (*A*) control. The peak at 700 nm in (*B*) stems from oxidized *c*.

namely that reduction of a_3^{3+} by *c* (probably by way of *a*) is a slow reaction. Under our conditions the rate is closer to that found by the latter authors. These authors (10) concluded from their experiments that copper is not significantly reduced in the first rapid phase of reduction. We find rapid disappearance of the EPR signal for copper and the 800-nm absorption in the reflectance spectra, as had been observed in this laboratory (1, 17), but this does not unambiguously indicate reduction of copper, and, as also pointed out by these authors, the conditions of our experiments are quite different, so that these results are not necessarily incompatible. Fig. 7 shows a plot of the changes observed in the EPR signal intensities of the detectable components at 6 and 100 msec when reduced enzyme is reoxidized with O_2, ferricyanide (4 equivalents per heme), or *c* (2 equivalents per heme). The corresponding reflectance spectra are shown in Fig. 8. It is seen that both ferricyanide and *c* produce intense signals at $g = 6$, but do not substantially restore the band at 655 nm, whereas within 6 msec oxygen converts all components back to their oxidized state.

It is apparent from the limited data shown above and from other experiments that we have performed that neither the intensity of the signal at $g = 3$ nor of that at $g = 6$ is directly related to the intensity of the 655-nm absorption. This indicates that this absorption band represents a separate species. On the other hand, there is an inverse relationship between the appearance of the $g = 6$ and the $g = 3$ signals. The $g = 6$ signal appears as the $g = 3$ signal disappears, and we have never seen strong $g = 3$ and $g = 6$ signals simultaneously.

DISCUSSION

Although the interpretation offered here appears to be in agreement with recent results, as well as with the older spec-

trophotometric data, and also postulates what had been intuitively inferred for many years, namely that a_3^{3+} is a high-spin heme compound, we would like to emphasize that our proposal is still too limited to account for all detailed observations, e.g., the identity of all EPR-detectable heme species and their quantitative relationships. In the presence of strong ligands such as CO or azide, additional complications are introduced. According to measurements of magnetic susceptibility at room temperature (22) and at 1.5–77°K (Moss, Hartzell, and Beinert, to be published), the paramagnetism of *c* oxidase in the oxidized state is not sufficient to account for the presence, per minimal functional unit, of one heme in the high-spin state. It is therefore likely that there is spin coupling between two components of the enzyme. This has been proposed previously, and it was considered likely that the undetectable components, namely part of the copper and one heme, are involved (1, 23). Considering the few other enzymes that have the capability of rapidly producing water from O_2, namely the blue copper oxidases (24), we find it attractive to compare the high-potential heme–copper pair of *c* oxidase undetectable by EPR, with the high-potential copper pair of the blue oxidases undetectable by EPR. Although recently much emphasis has been placed on heme–heme interaction in the enzyme (see refs. 4 and 8), it is probably not justified at this stage to single out heme–heme interaction over other possible interactions. It appears to us from our own experimentation and from results reported by others that there are multiple interactions of various strengths between the components of *c* oxidase, the very situation which makes it almost—if not entirely—impossible to elicit, by certain additions, an unambiguously specific response of one of the components.

It has been argued that studies on the purified enzyme are of very limited value, as changes may have occurred on isolation. We are aware of this, but consider pursuit of work on the purified enzyme important at this stage of our knowledge for the following reasons: (*1*) Obviously the isolated enzyme produces water from oxygen within microseconds, one of the principal capabilities of the enzyme in tissue. It is extremely unlikely that information obtained on how this is brought about by the purified enzyme will be useless for understanding the function of the enzyme in its natural environment. (*2*) The sensitivity of our measurements is increased an order of magnitude or better when we work with the purified enzyme, because of the concentrations that can be used. Many details can thus be recognized that one would not even become aware of in work with the enzyme in mitochondrial particles. When such details and conditions, under which they can be observed, are worked out, only then will it be possible to search for similar phenomena in the particulate enzyme. Thus, for instance, the fact that there is a great number of species of all the heme signals of the enzyme, depending on ionic environment, pH, dissolved gas, kind and quantity of oxidant or reductant present, and time of reaction, cannot be recognized in work on particles because of lack of resolution. Since these species have *g* values shifted by as much as 30 G and substantially different signal shapes‡, it is hazardous to derive quantitative information from merely observing signal height. Furthermore, the assumption that the EPR spectra observed with isolated mitochondria or particles, as opposed to those of the purified enzyme, represent the enzyme "*in situ*" (see ref. 4) is not valid. We have observed that the shape and *g* values of the heme signals depend on the medium of particle isolation and other conditions, as mentioned above. EPR spectra of *c* oxidase in tissue obtained quasi *in vivo* by freeze clamping are quite different from those seen in particles after isolation from the same animal species‡.

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