Biochimica et Biophysica Acta, 423 (1976) 339–355
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BBA 46992

KINETIC STUDIES ON CYTOCHROME *c* OXIDASE BY COMBINED EPR AND REFLECTANCE SPECTROSCOPY AFTER RAPID FREEZING

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(Received May 1st, 1975)
(Revised manuscript received November 3rd, 1975)

SUMMARY

1. Techniques and experiments are described concerned with the millisecond kinetics of EPR-detectable changes brought about in cytochrome *c* oxidase by reduced cytochrome *c* and, after reduction with various agents, by reoxidation with O_2 or ferricyanide. Some experiments in the presence of ligands are also reported. Light absorption was monitored by low-temperature reflectance spectroscopy.

2. In the rapid phase of reduction of cytochrome *c* oxidase by cytochrome *c* (< 50 ms) approx. 0.5 electron equivalent per heme *a* is transferred mainly to the low-spin heme component of cytochrome *c* oxidase and partly to the EPR-detectable copper. In a slow phase (> 1 s) the copper is reoxidized and high-spin ferric heme signals appear with a predominant rhombic component. Simultaneously the absorption band at 655 nm decreases and the Soret band at 444 nm appears between the split Soret band (442 and 447 nm) of reduced cytochrome *a*.

3. On reoxidation of reduced enzyme by oxygen all EPR and optical features are restored within 6 ms. On reoxidation by O_2 in the presence of an excess of reduced cytochrome *c*, states can be observed where the low-spin heme and copper signals are largely absent but the absorption at 655 nm is maximal, indicating that the low-spin heme and copper components are at the substrate side and the component(s) represented in the 655 nm absorption at the O_2 side of the system. On reoxidation with ferricyanide the 655 nm absorption is not readily restored but a ferric high-spin heme, represented by a strong rhombic signal, accumulates.

4. On reoxidation of partly reduced enzyme by oxygen, the rhombic high-spin signals disappear within 6 ms, whereas the axial signals disappear more slowly, indicating that these species are not in rapid equilibrium. Similar observations are made when partly reduced enzyme is mixed with CO.

5. The results of this and the accompanying paper are discussed and on this basis an assignment of the major EPR signals and of the 655 nm absorption is proposed, which in essence is that published previously (Hartzell, C. R., Hansen, R. E. and Beinert, H. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2477–2481). Both the low-spin ($g = 3$; 2.2; 1.5) and slowly appearing high-spin ($g = 6$; 2) signals are at-

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tributed to ferric cytochrome *a*, whereas the 655 nm absorption is thought to ari from ferric cytochrome a_3, when it is present in a state of interaction with EP undetectable copper. Alternative possibilities and possible inconsistencies with th proposal are discussed.

INTRODUCTION

In a preceding publication [1] we have described qualitative and quantitativ aspects of the principal EPR signals observed with preparations of cytochrome oxidase. The assignment of the various resonances to components of the enzyme wa briefly discussed. Although some of these signals are seen under equilibrium as we as rapid reaction conditions [1, 2], it was pointed out that some species are typica of certain reaction times. The present publication presents results of studies by low temperature EPR and optical reflectance spectroscopy on samples of cytochrome oxidase obtained during reduction or oxidation reactions by the rapid mix-freeze quenching technique [3]. We have previously described observations by this techniqu for some of the more obvious reaction conditions [4–6]. This report essentiall confirms these and is an extension to more specialized conditions. These experiment have a particular bearing on the assignment of resonances and optical absorptio bands of the enzyme.

METHODS AND MATERIALS

The experimental techniques and materials used were described in preceding papers [1, 2] with the exception of those pertaining to the rapid-freeze technique. This technique, as originally described by Bray [3] has been modified and developed independently in a few laboratories [7–9]. Two principal modifications were introduced in our laboratory. (1) An electric ram system furnishes the force driving the syringes [7] and with the present version the ram can be stopped and actuated again to expel the mixture generated in the first phase. (2) Special precautions were taken to exclude exposure of the solutions to oxygen. For this purpose the tonometer, syringe, mixing chamber and hose system were modified as well as the receiving system in which the spray of the reaction mixture was collected. The soft plastics polyethylene and teflon were replaced with hard nylon (nylon 66), epoxy cement and lucite, whenever feasible, and viton 0-rings were used instead of teflon, where resilient seals were required. The mixing chambers, which were of the general design published previously [10] had a separate channel entering in the center of the cylindrical chamber from the top, at right angles to the jet system. This channel was closed by a screw with a conical teflon tip. The tip was tightly sealed during operation but was loosened during preparation for a ‘shot’ so that nitrogen could be passed through the chamber, attached nylon reaction hose, and spray nozzle. The whole syringe assembly was kept in a lucite compartment which was flushed with prepurified nitrogen. The collecting tubes containing the cold isopentane were mounted in a metal dewar container where they were kept at the proper temperature by a stream of cold nitrogen gas automatically controlled essentially as described [11]. This dewar assembly was then moved on a rail under the lucite box housing the syringe,

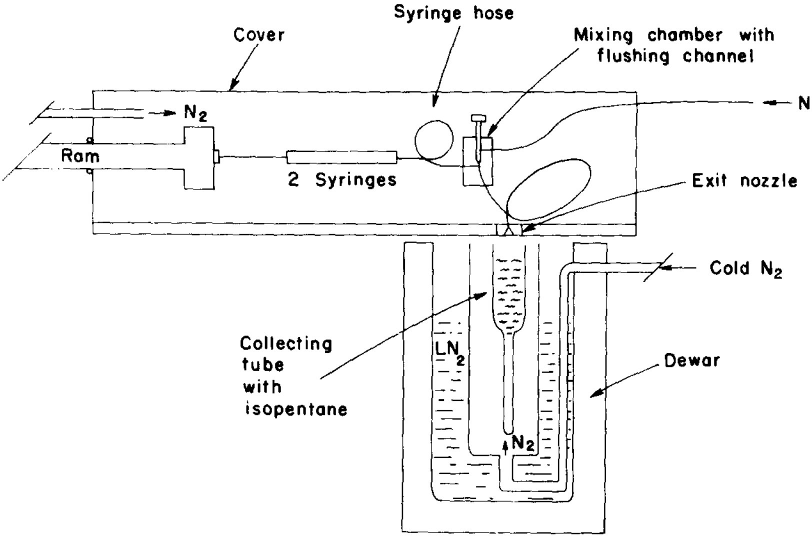

Fig. 1. Schematic illustration of the ram-syringe- and collecting system used for anaerobic rapid freeze-quenching. For other details and similar systems, see refs 3, 7 and 9.

chamber- and nozzle assembly, so that the space between the spray nozzle and the receiving funnel-tube was almost closed in. This space was rapidly filled by the nitrogen emerging from the dewar assembly (cf. Fig. 1). Nylon hoses were purchased from the Polymer Corporation, Reading, Pa. As in our previous publications concerned with rapid freeze-quenching, the reaction times reported are those calculated from the dimensions of the system and ram speed, not considering the time required to stop the reaction. The rapid reaction system was tested for anaerobic conditions by the use of cytochrome *c* oxidase itself. The oxidation of the enzyme by molecular oxygen is too fast to be resolved by our system. Since a number of reductants react only very slowly compared to the oxygen reaction, it was feasible to use excess reductant to obtain the completely reduced enzyme. This is preferable to using partly reduced enzyme, as it has been found that some species occurring in partly reduced preparations are only slowly reoxidized (see below). The enzyme was reduced over several hours with a 3-fold excess of NADH (mol/mol heme *a*) in the presence of cytochrome *c* at 1/100 the heme *a* concentration of the oxidase. While all precautions enumerated above were taken, control shots were made by directly connecting the hose of the syringe containing the reduced oxidase to the nozzle. Other conditions were then tested, as summarized in Table I. The appearance of the signal at $g = 3$ and the copper signal were used as indicators of reoxidation, since these components respond very rapidly to the presence of oxygen. It is apparent from Table I that some reoxidation does take place even under the simplest manipulations, namely the control shot conditions given above. In all test experiments there was some unexplained scatter in the values, indicating that some sources of contaminating oxygen were not under our control. The following conclusions appear justified. There were no significant differences when the mixing chamber was interposed and when anaerobic buffer was mixed in from a second syringe. Use of longer reaction hoses (90 cm)

TABLE I

REOXIDATION OF LOW-SPIN HEME OF REDUCED CYTOCHROME *c* OXIDASE DURING RAPID FREEZING UNDER DIFFERENT CONDITIONS

Reaction time (s)	Length of reaction hose (cm)	Comments on special conditions**	Low-spin heme (g = reoxidized* (%)
< 0.005	0	directly from syringe to nozzle, no mixing chamber	4.7
0.006	1.7	mixed; start of experiment	4.8
0.006	1.7	mixed; end of experiment (2 h)	4.3
0.006	1.7	mixed; air between nozzle and receiving tube	17.5
0.230	90	mixed	7.0
600	90	mixed; double push mode	11.3
< 0.005	0	directly from syringe to nozzle, no mixing chamber	3.3
0.006	1.7	second entrance to mixing chamber plugged	1.3
0.006	1.7	mixed	3.0
0.230	90	second entrance to mixing chamber plugged	1.7
60	90	double push mode	2.3

* The enzyme concentration in the syringe was 0.2 mM for the first six experiments and 0.33 mM for the remaining experiments.

** "Mixed" indicates that anaerobic buffer was mixed with the enzyme from a second syringe via the mixing chamber. Otherwise no chamber was used or the second entrance to the chamber was plugged.

to produce times in excess of 230 ms did contribute oxygen, particularly when the mixture was held in the hose for several min. Apparently, this oxygen had been adsorbed on the inside wall of the hose. We tried to evacuate and precondition the reaction hoses with nitrogen without noticing any effect. As might be expected, considerable reoxidation occurred, when anaerobic conditions were not maintained between exit nozzle and collecting tube. Oxygen did not seem to enter the syringes to any significant extent during sitting with the reactants within the 2–3 hours of an experiment. We also examined the samples by low-temperature reflectance spectroscopy, since it was conceivable that some changes in the optical spectra might be detected before those of the EPR spectra occur. It appears this was not the case, as there was no detectable absorbance change in the Soret-, α-region, or 655 nm absorption under our conditions and with the time resolution of the apparatus. From a knowledge of the quantity of the components which are reoxidized and the enzyme concentration, a gross estimate of the quantity of oxygen that is apparently present can be made. According to this calculation 1 nmol of O_2, corresponding to about 0.1 μl of air, finds its way into the approx. 0.3 ml of reaction mixture. Considering that we have generally used the enzyme at a final concentration (after mixing) of 0.5 mM, this would mean that the ratio of mol heme/mol oxygen is approx. 150. It has been suggested that the presence of oxygen may effect a modification in cytochrome *c* oxidase, which changes the characteristics of its reduction by cytochrome *c* [12]. We wonder whether under our conditions, at a heme to oxygen ratio of 150, this is likely to occur, particularly since whatever oxygen is present is readily consumed in the presence of a reductant.

RESULTS

Reduction of cytochrome c oxidase

Fig. 2 shows the course of disappearance of the signals of the detectable components and oxidation of cytochrome *c* when 1 mol of reduced cytochrome c per mol of total heme *a* is added. The reaction with cytochrome *c* proceeds essentially as published previously (ref. 5, Fig. 14), although the special precautions concerning anaerobiosis, enumerated above, were not taken in the earlier work and resolution of the high-spin signal was not possible. Table II shows the balance of the oxidation states of the components in a number of experiments carried out at different pH values and different levels of cytochrome *c*. As in titrations [2], the extent to which the low-spin heme signal disappears increases with increasing pH. Likewise, the disappearance of the copper signal is least at low pH. Nevertheless, cytochrome *c* is rapidly and extensively oxidized at pH 6, indicating that under these conditions electrons are shifted toward the EPR-undetectable components. Experiments with

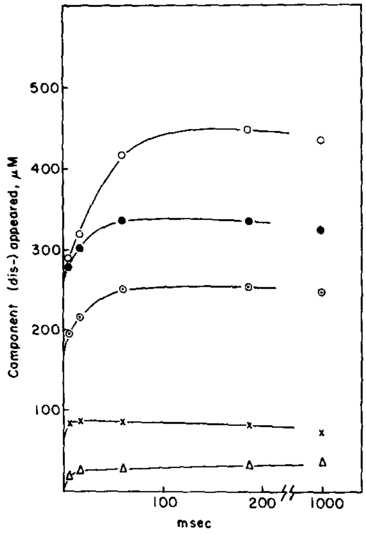

Fig. 2. Kinetics of EPR-observable changes during anaerobic reduction of cytochrome *c* oxidase by reduced cytochrome *c*. 1.16 mM enzyme was rapidly mixed with an equal volume of 0.9 mM cytochrome *c*. The abscissa shows reaction time at 17 °C after mixing and the ordinate the changes observed in the various EPR-observable components expressed as nmol/ml of the resulting mixture. The symbols indicate: ○, cytochrome *c* oxidized; ⊙, low-spin heme signal disappeared; ×, copper signal disappeared; ●, sum of low-spin heme and copper signals disappeared; △, axial high-spin signal appeared.

TABLE II

BALANCE OF OXIDATION-REDUCTION CHANGES ON RAPID REDUCTION OF CYTOCHROME *c* OXIDASE BY CYTOCHROME *c* AT DIFFERENT pH AND CYTOCHROME *c* TO OXIDASE RATIOS

These experiments were carried out in phosphate buffer at pH 7.4 or at pH 6.1 and 8.65, after adjustment of pH by 1 M acetic acid and Tris base, respectively. The concentrations of reactants given are those resulting after mixing equal volumes of anaerobic solutions. The concentrations of cytochrome *c* refer to the reduced form present. There was $< 3\,\%$ of the oxidized form present. The quantitative evaluation of the ferric high-spin signals was based on double integration as detailed in ref. 1. A correction was applied for the volume occupied by isopentane. For cytochrome *c* oxidase this correction factor is 2.1. For the copper and low-spin heme signals only relative comparisons of signal heights were made and the quantities were calculated on the basis that the low-spin heme ($g = 3$) represents 50 % of the total heme and the active copper, which is detectable by EPR, is present at a concentration 35 % of that of heme *a*.

pH	Cytochrome oxidase (μM)	Cytochrome *c* (μM)	*c*/*a* ratio	Reaction time (ms)	Cytochrome *c* oxidized (nmol)	Low-spin heme disappeared (nmol)	Cu disappeared (nmol)	High-spin heme appeared (nmol)	Low-spin heme+Cu disappeared (nmol)
7.4	612	608	1.0	6	305	180	85.5	27	265.5
7.4	612	608	1.0	100	439	252	135	40	387
7.4	612	1270	2.0	6	428	250	146	38	396
7.4	612	1270	2.0	100	558	276	171	42	447
6.1	400	550	1.38	6	315	112	39	74.5	151
6.1	400	550	1.38	25	342	143	57.5	81	200.5
6.1	400	550	1.38	100	425	149	54.5	105	203.5
6.1	400	550	1.38	1000	470	157	56	110	213
8.65	480	490	1.02	6	302	201	72.5	8.4	273.5
8.65	480	490	1.02	25	313	220	82.5	8.4	302.5
8.65	480	490	1.02	100	316	220	85.5	15	305.5
8.65	480	490	1.02	1000	358	220	84	17	304

levels of cytochrome *c* higher than those used here suffer from difficulties of evaluation, since EPR as well as optical absorption of the low-spin *c* and *a* hemes overlap. The following observations are significant. The initial phase of rapid changes ends after 50 to 100 ms. Relatively slow intramolecular electron transfer processes seem to follow. Approx. 1 reducing equivalent is transferred per oxidase unit (2 heme; 2 Cu) very rapidly (6 ms) with 1 or 2 mol of cytochrome *c* per mol of total heme *a*; this is largely accounted for by the disappearance of the low-spin heme and copper signals, so that in this instance signal disappearance may indeed be equivalent to reduction. There follows more extensive oxidation of cytochrome *c*, particularly at the higher concentration of cytochrome *c*. The low-spin heme signal disappears most rapidly, closely followed by part of the copper. However, as indicated by the titration experiments [2], where no disappearance of the copper signal is observed initially, copper is again reoxidized, apparently by internal electron transfer after initial reduction. Thus we may in fact be observing a quasi-steady-state level of the copper signal. At early reaction times, under our conditions, we have never observed the situation where the low-spin heme but no copper was reduced [12]. With cytochrome *c* as reductant, as with dithionite, no rhombic high-spin signal is observed before approx. 1 s of reaction. At that time it accounts for approx. 20 % of the integrated intensity of the axial high-spin signal. The absorption band at 655 nm disappears only slowly. There is no significant disappearance of this band within 1 s (< 20 %); there is an approx. 25 % decrease at 1 min. Experiments on the reduction of the enzyme by dithionite have been published previously [6]. In these experiments the high-spin signal ($g = 6$) does not show a rhombic component (cf. Fig. 11A, ref. 1), except after approx. 10 min of reaction and the rhombic component amounts to at most 50 % of the heme represented in the axial signal. Over this same time range the absorption band at 655 nm disappears very slowly. As on reduction with cytochrome *c* the low-spin signal ($g = 3$; 2.2; 1.5) disappears faster than the copper signal except that the rates are several hundred times slower with dithionite. Typical half times of disappearance for these signals are 0.3 and 2 s for the heme and copper signals, respectively, at 16 °C, 0.5 mM heme *a* and 7 mM dithionite.

Reoxidation of completely reduced cytochrome c oxidase

In the absence of an excess of reduced cytochrome *c* the course of reoxidation of the enzyme by oxygen is too rapid to be followed by the rapid-freeze technique in its present version. All signals are restored to their original levels and the high-spin signal at $g = 6$ is minimal. Likewise, the optical absorption bands, including that at 655 nm, are typical of the normal oxidized form. However, a striking observation is that immediately on reoxidation with oxygen the so far unassigned signals at $g = 1.69$ and 1.78 [1] are unusually large, whereas they are often absent or small in samples as obtained in the purification procedure or after storage. They are not seen on reoxidation with ferricyanide. This might be related to auto-reduction of some component during storage or other handling or to other secondary rearrangements within the enzyme following sudden reoxidation of a reduced preparation.

Fig. 3 illustrates the results obtained when cytochrome *c* oxidase is reduced with an approximately two-fold excess of reductant and then exposed to an excess of oxygen. In a typical example, an enzyme preparation (0.9 mM) that had been reduced with 2 equivalents of cytochrome *c* per heme *a* and an equivalent amount of ascorbate was

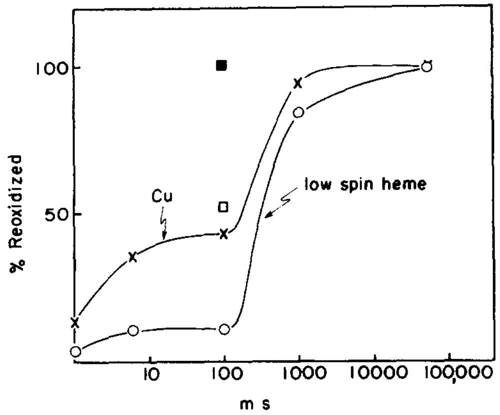

Fig. 3. Kinetics of reoxidation of completely reduced cytochrome *c* oxidase in the presence of a excess of reductant and oxygen. 0.9 mM enzyme was reduced anaerobically with 1.8 mM reduce cytochrome *c* in the presence of 0.9 mM ascorbate. This solution was rapidly mixed with an equ volume of 0.1 M phosphate buffer of pH 7.4 saturated with oxygen at 18 °C (1.4 mM). One sampl was mixed anaerobically with an equal volume of 4 mM potassium ferricyanide in phosphate buffe The abscissa shows time in ms on a logarithmic scale, the ordinate % oxidized with respect to fully oxidized (O_2) blank on the assumption that reappearance of the respective signals indicate reoxidation. The symbols indicate: ○, low-spin heme; ×, copper, reoxidized with oxyge □, low-spin heme; ■, copper, reoxidized with ferricyanide.

rapidly mixed with buffer, 1.4 mM in oxygen. In this case the low-spin heme and th copper signals were only partly restored (Fig. 3) and only weak axial (no rhombic high-spin signals were observed. Reduced cytochrome *c* and partly reduced heme were clearly indicated in the Soret and α absorptions of the reflectance spectra, whil at the same time the 655 nm band was as intensive as in the oxidized control (Fig. 4 This indicates a situation where electron transfer from reduced cytochrome *c* to th low-spin heme and EPR-detectable copper is faster than subsequent intramolecula transfer to the component(s) detected at 655 nm. This component has obviousl communicated rapidly with oxygen and (if it is an electron carrier as we believe) ha given up electrons to oxygen. It is known that communication between differen units* (or maybe blocks of units) is much slower than intra-unit electron transfe [5, 13]. This type of experiment clearly indicates that low-spin heme and EPR detectable copper are on the electron-receiving, and the 655 nm components(s) on th electron-donating end of the unit.

With ferricyanide (4 mM) as oxidant, the absorption at 655 nm is not restore even at 1 min, whereas the maximal rhombic high-spin signal ever seen by us is pro duced within 0.1 to 1 s. In experiments where 0.44 mM enzyme had been reduced b 0.85 mM cytochrome *c* in the presence of an equal amount of ascorbate, reoxidatio with 4 and 8 mM ferricyanide led to signals at $g = 6$ that accounted for a total o 40–45 % (axial 10 %, rhombic 30–35 %) of the total heme iron, while the low-spi heme was approx. two thirds reoxidized, thus accounting for 30–35 % of the tota heme. An interesting feature of ferricyanide reoxidation is that the copper signal als

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* As a unit, we consider the minimal functional unit as defined in ref. 5, namely a structur containing cytochromes *a* and a_3 and both the EPR-detectable and -undetectable copper.

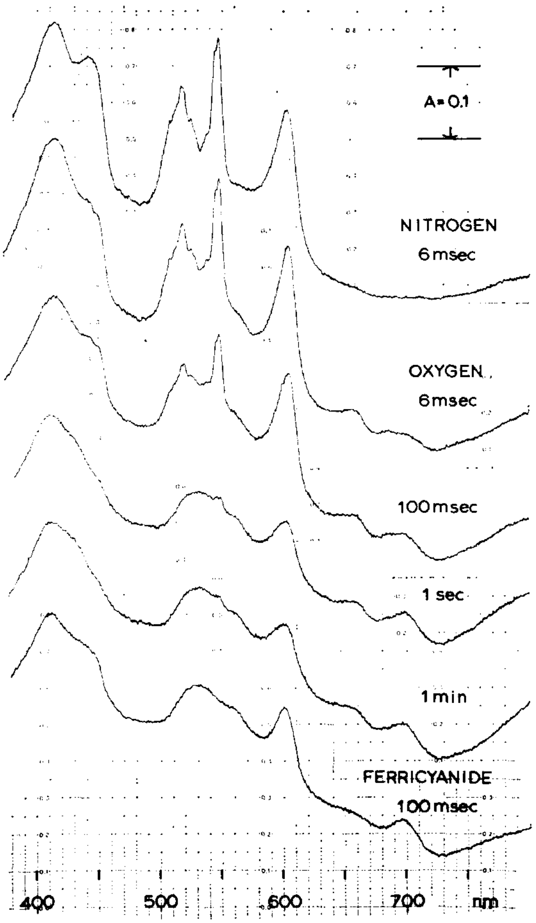

Fig. 4. Low-temperature (100 °K) reflectance spectra recorded on samples from the experiment of Fig. 3 (reduced blank, 6 ms, 100 ms, 1 s and 1 min after mixing with oxygenated buffer, and 100 ms after mixing with 4 mM ferricyanide solution).

increases beyond the magnitude observed in the oxidized preparation as isolated. After reoxidation with ferricyanide, the EPR-detectable copper can thus account, within integration error, for 50 % of the total copper present in the enzyme, whereas we have never seen an increase of more than 10 % on addition of ferricyanide to the enzyme as isolated. Also, on reoxidation with ferricyanide we have always detected a free radical signal in the first 100 ms of the reaction. However, the quantity of unpaired spin represented in this signal amounts to only 0.25 % of the heme. The significance of this signal is therefore questionable.

Reoxidation of partly reduced enzyme

Of particular interest was the behavior of the signal of the high-spin intermediate ($g = 6$) on reoxidation. Since this signal is only of significant magnitude after partial reduction, experiments were conducted in which the enzyme, partly reduced with cytochrome *c*, was reoxidized by oxygen. When oxidase is partly reduce with cytochrome *c* under equilibrium conditions at pH 7.4, a strong signal at $g =$ with axial and rhombic components is always observed. On reoxidation with oxyge the rhombic signal is eliminated within 6 ms, whereas the axial component of th signal is still present at one half the original level at that time. The axial and rhombi components are therefore not in rapid equilibrium. However, when an axial signal a $g = 6$ without rhombic component (type of ref. 1, Fig. 11A), is generated by rapi reduction with reduced cytochrome *c*, and oxygenated buffer is mixed in from a thir syringe, this initial axial signal persists for several seconds.

It was this type of observation, where certain signals at $g = 6$ are not re sponding readily to oxygen, which led us previously [14] to generalize that partl reduced cytochrome *c* oxidase is not able to react effectively with oxygen. The ob servation is confirmed, but since the initial signals at $g = 6$, which are refractory t oxygen, account for only 3–4 % of the total heme, it is correct to say that only i those cytochrome *c* oxidase units that are responsible for these signals is the origina oxidized state not readily restored by oxygen.

Reactions in the presence of carbon monoxide and other ligands

Carbon monoxide. On partial reduction in the presence of CO and in th absence of light, only small signals at $g = 6$ are seen. When the experiment describe above, in which enzyme partly reduced with cytochrome *c* was mixed with oxygenate buffer, was repeated with buffer saturated with CO instead of oxygen, the usua rhombic signal observed on reduction with cytochrome *c* (ref. 1. Fig, 14A and Tabl VI) was replaced by the rhombic component typical of the presence of CO (ref. 1 Fig. 12a and Table VI) within 6 ms, whereas the axial signal retained its shape, bu disappeared with a half time of 15 ms, again indicating that the components givin rise to the various high-spin signals are not in rapid equilibrium. Within 1 s the tota signal at $g = 6$ was diminished to a very low level. The significance of this residua signal is questionable [15]. The observation reported here, that on oxidation-reductio the rhombic and axial high-spin signals are not in very rapid equilibrium (< 5 ms does not contradict the finding mentioned in the preceding paper [2] that there is apparently a very fast conversion between ionization states related to these signals. Any single cytochrome *c* oxidase unit will have either a rhombic or an axia high-spin component, which may be rapidly converted to the other form when the pH is changed, but for oxidation-reduction different units would have to interact an exchange electrons which is known to be a relatively slow process [5, 13]. In experi- ments in which enzyme reduced in the presence of CO was mixed with oxygen, the low-spin heme and copper signals reappeared slowly, as expected. These signals a well as the 655 nm band returned in the time range of seconds not ms as in the absence of CO.

Chloride. It has been reported in the preceding paper that chloride changes the shape of the low-spin heme signal [1]. When cytochrome *c* oxidase was reduced with a dithionite/KCl mixture [16] and then mixed with oxygenated buffer it was observed that the signal at $g = 3$, which appeared instantly, initially had the normal shape (ref. 1, Fig. 3) and only with a half time of approx. 5 ms shifted upfield and assumed the chloride shape (ref. 1, Fig. 3). This indicates that Cl^- does not bind, or at least not in the same fashion, to the reduced as it does to the oxidized enzyme, and that electron loss from the heme is apparently faster than the subsequent rearrangement of its environment which admits Cl^-.

Cyanide. No systematic study was made with cyanide. When the reduced (dithionite) enzyme was rapidly mixed with KCN solution and thereafter with oxygen, the rate of the appearance of the low-spin heme and copper signals and the 655 nm absorption was retarded: after 30 ms of exposure to 10 mM KCN, followed by oxygen for 6 ms, all features had returned to their original values, whereas when the exposure to cyanide had been 1 s, only about 50 % of the low-spin heme signal at $g = 3$ and of the copper signal had returned and the 655 nm band was only approx. 25 % developed. This observation is in general agreement with the rates reported for the reaction of reduced enzyme with cyanide [17]. In neither sample, whether exposed to cyanide for 30 or 1000 ms, was there any signal seen at $g = 3.6$, a signal which is thought to originate from the cyanide complex of cytochrome a_3 [18]. Weak signals at $g = 3.6$ were, however, observed when reduced samples, exposed to cyanide as above, were reoxidized with ferricyanide.

DISCUSSION

In the preceding paper, we summarized from a large number of kinetic experiments mainly those observations which we think may be useful in arriving at plausible assignments of optical as well as EPR absorptions to specific components or states of the enzyme. Since we find more optical and EPR features than the number of known total components of the enzyme, it becomes important to search for relationships between some of these features in order to simplify the picture. With this in mind we will now discuss the individual detectable components of cytochrome *c* oxidase and the conclusions or inferences concerning them, to which the work described in this series of papers [1, 2, 6] leads us.

Copper

The observations that the copper signal during reductive titrations initially increases somewhat rather than decreases and that this signal on reoxidation with ferricyanide may increase by as much as 25 % indicates that 50 % of the total copper, i.e. one of the 2 copper atoms in the enzyme, can in fact be detected under certain conditions. This seems to remove some doubts as to the validity of integrations of the copper signal of the resting enzyme, which consistently indicated [1, 2] that only 30–40 % of the total copper is accounted for in this signal. The unusual shape and *g* values of the copper signal have raised the question as to whether it might in fact represent an interacting pair of copper atoms in the enzyme [5, 19–22]*. The unusual shape and low intensity might then be explained by magnetic dipolar interaction. Since no copper signals different from that generally seen in cytochrome *c* oxidase have ever been observed on oxidation reduction, this would imply that the copper pair undergoes oxidation-reduction in concert. This does not seem compatible with

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* Although there are few explicit suggestions in the literature, this question has been raised repeatedly in discussions and at meetings.

the results of the stoichiometries [2] and midpoint potentials observed or calculate from titrations [23]. The possibility that the observed and calculated stoichiometri may not be valid because some damage may have occurred in the preparations used and only a fraction of the copper may be in the paired form, is again unlikely becaus of the absence of significant amounts of other copper signals. In rapid reduction an reoxidation experiments, the copper signal closely follows that of the low-spin hem ($g=3$; 2.2; 1.5). The behavior of the absorption band at 830 nm in reflectance mea surements has always been found to parallel that of the copper signals. (Note tha baseline changes are minimized and long wavelength bands intensified in reflectanc spectra.) Although minor changes in the shape of the copper signal can be observe under some conditions, we are unable to rule out the possibility that they might b due to changes in shape or intensity (absolute or relative to the signal of active copper of underlying signals of heme components, inactive copper or free radicals.

Low-spin heme ($g = 3$; 2.2; 1.5)

This signal appears to represent the component of the enzyme that respond initially to reductants, e.g. it disappears 65(75)% within 6 ms with 1(2) mol of reduced cytochrome *c* per heme *a* in the range of 0.5 mM concentrations. Its behavior can therefore best be equated to that of the component termed cytochrome *a* in earlier kinetic studies carried out by spectrophotometry [24–26]. On reoxidation with oxygen, this signal likewise responds rapidly and its size can be taken as an indicator of the presence of oxygen in supposedly anaerobic preparations. Upon reoxidation with 2 mM ferricyanide, this signal returns more slowly, so that it is 70 % restored at 100 ms.

High-spin heme ($g = 6$; 2)

The identity of the various species of this signal poses a most difficult problem. The different species observed and their characteristics were described in a previous publication [1] of this series. The rapid reduction experiments show that up to 1 s with reduced cytochrome *c*, and in the first minutes with dithionite, there is very little if any rhombic component of the high-spin signal present. Under these conditions at pH 7.4, the amount of heme represented in the axial signal is rather small (< 5 % of the total heme) and even less at high pH. One may therefore question the significance of the slowly appearing high-spin signals for the catalytic process. The largest rhombic signals were formed within the first second on reoxidation of reduced (cytochrome *c* or NADH) oxidase with 4–8 mM ferricyanide, when they can account for as much as 30–35 % of the total heme. At this time an axial signal is also present amounting to approx. 10 % of the heme; situations can therefore be realized where over 40 % of the heme is accounted for in the high-spin signal while about 30–35 % is present in the low-spin form. Experiments yielding a high recovery of heme in the high- and low-spin signals were described by Wever et al. [15]. Enzyme was partly reduced in the presence of CO, reoxidized with ferricyanide and then frozen in bright light. In our attempts to repeat these experiments we were never able to reach a yield of more than 15 % of the total heme in the rhombic high-spin signal, only half of what we observe on rapid reoxidation with ferricyanide, as detailed above. These findings have a bearing on an assignment of EPR signals which we had proposed previously [6], namely that at least one of the high-spin species represented in the signals at $g = 6$ may originate from the same heme component of the enzyme that gives rise to the low-spin signal at $g = 3$. This would result from initial reduction (decrease in $g = 3$) and subsequent reoxidation (increase in $g = 6$) of this same component by other EPR-undetectable electron acceptors in the enzyme. Support for this suggestion was offered by the correspondence of the disappearance of $g = 3$ with the emergence of $g = 6$ and the observation that strong signals at both $g = 3$ and $g = 6$ never appeared simultaneously, i.e. the integrated intensities of both signals together had never been found to exceed 50 % of the total heme present. The situation arising on rapid reoxidation of cytochrome *c* oxidase by ferricyanide described above, when approx. 70–80 % of the heme iron seems to be accounted for in the low- and high-spin signals together, certainly throws doubt on this earlier suggestion. However, we cannot consider the reported experiments as entirely decisive. Firstly, a possibility exists of a relatively large error in the absolute values derived from the integrations, particularly of the high-spin signal; secondly, at least 2 high-spin species, axial and rhombic, are present, which do not have to originate from the same heme. Thus our earlier suggestion could still be valid for one of these species. It has also been considered that either heme may be represented, under certain conditions, in the low-spin signal [21, 27]. The restriction that the intensities of the low- and high-spin signals together should not exceed 50 % of the total heme is removed for all conditions for which none of the hemes is assumed to be EPR silent.

Absorption band at 655 nm.

We have previously drawn attention to this absorption band [6], which is very prominent in the reflectance spectrum of the fully oxidized enzyme (cf. Fig. 4). While the 655 nm band disappears, the absorption at 605 nm rises, which is, however, no specific correlation, as the reduced forms of both hemes probably absorb in the 600 nm region. There appears to be a direct correlation between the 655 nm absorption and the broad band at 550 nm, observed in the oxidized form (cf. Fig. 4 of ref. 2) and an inverse relationship to the absorption band at 444 nm, as pointed out previously [6] and shown here in Fig. 4. It is generally agreed that cytochrome *a* has a split Soret band with maxima at low temperatures at 442 and 447 nm [28], whereas the single absorption maximum of cytochrome a_3 is located at 444 nm (cf. Fig. 4). The 655 nm band exhibits kinetics different from any of the EPR-detectable components of the enzyme. However, the kinetics of appearance of the rhombic ferric high-spin signal appear to match the kinetics of disappearance of the 655 nm absorption, within the accuracy of our determinations.

Assignment of EPR and optical features.

With this information and these relationships in mind, we will now consider the alternatives available for the assignment of the 655 nm absorption: the low spin heme ($g = 3$; 2.2; 1.5) and detectable copper components show no relationship to the 655 nm absorption, since on reduction of the enzyme the low-spin heme and copper signals disappear much faster than the 655 nm absorption, and on reoxidation with ferricyanide these signals reappear rapidly, whereas the 655 nm band does not. Furthermore, experiments of the type shown in Figs 3 and 4 show that there are conditions of reoxidation when the 655 nm band may be maximal, while the low-spin heme and copper signals are only 10 and 35 % developed, respectively, and, finally, i the enzyme oxidized by oxygen all three features are fully developed. Thus if neithe the detectable copper nor the low-spin heme ($g = 3$) is related to the 655 nm absorp tion, and if the kinetic evidence that the low-spin heme represents cytochrome *a* i accepted, then the choice is narrowed to the undetectable copper and the other hem component, cytochrome a_3. If this heme component were represented by the rhombi high-spin signal at $g = 6$, the 655 nm absorption could not at the same time be property of this heme because of the inverse kinetic relationship shown by these tw features. If, however, the major high-spin signal were attributed to ferric cytochrom a_3, it would be necessary to assign the 655 nm absorption to the undetectable coppe or to a state of interaction between this copper and cytochrome a_3, with only th copper becoming reduced when the 655 nm band disappears and the high-spin signal appear. The possibility that cytochrome a_3 is not reduced as the 655 nm absorptio disappears is not supported by the observed inverse relationship between the absorption at 655 nm and that at 444 nm. The rise of the 444 nm absorption during disappearance of the 655 nm absorption indicates that the heme component involved is indeed reduced; in other words, this heme is not likely to appear at the same time in quantity as ferric high-spin form. Support for the assignment of the 655 nm absorption also stems from the effects of CO on this band as detailed previously [2, 6]. In the presence of reductant and CO, this band is more readily abolished than in the absence of CO. Furthermore, it has been shown by Gibson et al. [24] that, when cytochrome *c* oxidase is reduced in the presence of CO, the appearance of photosensitivity is a slow process. It is generally agreed that appearance of photosensitivity is an indicator of the formation of ferrous cytochrome a_3, which combines rapidly with CO. As shown previously [6] and in this paper, the disappearance of the 655 nm absorption on reduction is also a slow process in purified cytochrome *c* oxidase.

These considerations, therefore, lead us back to our previous suggestion, that the total of our observations on kinetics and equilibrium behavior is best explained by the following assignments:

(1) The low-spin ferric heme signal ($g = 3$; 2.2, 1.5) represents ferric cytochrome *a* in the original fully oxidized form of the enzyme. In this state cytochrome *a* has a midpoint oxidation-reduction potential of sufficient magnitude and appears also to be the kinetically favored electron acceptor so that it is completely reduced by one cytochrome *c* per heme *a* at pH 7.4.

(2) The high-spin ferric heme signal at $g = 6$, with a strong rhombic component, which emerges after seconds or minutes of reduction (depending on the reductant) and disappears within milliseconds with oxygen, also represents ferric cytochrome *a*, but in a form of the enzyme in which cytochrome a_3 or the undetectable copper or both have been reduced with reoxidation of cytochrome *a*. In this state cytochrome *a* has a considerably lower midpoint oxidation-reduction potential [21].

(3) The absorption at 655 and 550 nm represents ferric cytochrome a_3 or, more likely (as discussed below), a state of the enzyme in which ferric cytochrome a_3 is coupled to the undetectable copper.

(4) The Soret bands at 442 and 447 and that at 444 nm, represent ferrous cytochromes *a* and a_3, respectively, as previously proposed [28].

These assignments not only provide a plausible explanation of our own observations but, as we see it, would also be compatible with kinetic data published by

Gibson et al. [24, 25], Andréasson et al. [12] and proposals put forth most recently by Nicholls and his collaborators [29, 30]. Andréasson et al. [12] concluded from their experiments that copper was not reduced in the initial fast reaction period ($<$ 100 ms) with cytochrome *c*, whereas we definitely see rapid disappearance of part of the copper signal. We do not consider this a serious discrepancy, as under our conditions, at one hundred times the concentration used by these authors, we certainly do not observe a second order reaction between cytochromes *c* and *a* ($g = 3$) with a rate constant as measured by these authors, so that in the experiments of Andréasson et al. [12] the fast reaction phase may well be more cleanly separated from the slow one, whereas in our experiments they tend to overlap. Nicholls [29] in fact concludes from his experiments that cytochrome *a* initially has a high midpoint potential as long as cytochrome a_3 is oxidized, which drops by 120 mV when cytochrome a_3 has been reduced. Nicholls and Petersen [30] suggest that of the two high-spin signals seen at intermediate stages in titrations, namely the axial and rhombic signals, one may represent cytochrome *a* and the other cytochrome a_3. While this possibility deserves consideration, we rather think that the weak axial ferric high-spin signal, which appears immediately on reduction and resists reoxidation by oxygen may well be a feature of a fraction of cytochrome a_3 and may even originate from non-functional units. The small quantity of heme represented in this signal makes any decisive experimentation difficult. Concerning the presence of two high-spin signals, viz. an axial and a rhombic one, we consider the possibility that one may be characteristic of cytochrome oxidase units in which both cytochrome a_3 and the undetectable copper are reduced and the other may represent such units in which only one of these components of high midpoint potential is reduced. It is also possible or even likely that the broad rhombic signal that can be elicited by illumination of cytochrome oxidase, which had first been reduced in the presence of carbon monoxide and then reoxidized by ferricyanide [15], is due to ferric cytochrome a_3. When this broad rhombic signal is observed, there is no significant absorption at 655 nm. Thus, if the rhombic signal generated by light in the presence of CO indeed represents cytochrome a_3, this would mean that the 655 nm absorption is not a characteristic of ferric cytochrome a_3 per se; for this reason we have suggested above that the 655 nm absorption is a feature of a special state of interaction between cytochrome a_3 and copper as it occurs in the resting, oxidized form of the enzyme.

An alternative assignment that has been proposed [5] is that the low-spin heme signal indicates cytochrome *a* and the major high-spin signals ferric cytochrome a_3. Such an assignment finds support in the behavior of the high-spin signal on addition of ligands and from the experiments on dissociation of the CO complex by light when strong rhombic ferric high-spin signals appear. Such an assignment, however, is hard to reconcile with the observed behavior of the 655 nm and 444 nm absorption and with the notion that it is the heme component of high midpoint potential, whose midpoint potential is further raised in the presence of CO, whereas the high-spin signals clearly represent components of low midpoint potential [21].

Our assignments seem also compatible with most observations of van Gelder et al. [27], although our interpretation of the 655 nm absorption and its relationship to the EPR-detectable components would exclude the notion that cytochromes *a* and a_3 are indistinguishable in the oxidized form of the enzyme. A discrepancy observed in the value of the mid-point potential for cytochrome *c* relative to the values for the cytochrome oxidase components was mentioned in the accompanying paper [2].

The assignment of the EPR signals which we prefer, as outlined above, rests mainly on the assumptions (1) that the 655 nm absorption represents a component of the majority of cytochrome *c* oxidase units present in a preparation and not of a minority and (2) that the observed rise of the 444 nm band under certain conditions of reduction does indeed indicate reduction of cytochrome a_3. We are not aware of any contradictory information on the first assumption and the second assumption has been generally accepted for many years [26, 28].

The observation most difficult to reconcile with our preferred assignment is that made on reoxidation with ferricyanide, when under some conditions large signals at $g = 3$ and at $g = 6$ were observed simultaneously, accounting in their combined intensities for more than one heme group per functional unit (aa_3, 2 Cu). However, it has been observed in independent coulometric work that titrations with ferricyanide are not reversible and do not seem to follow a readily interpretable mechanism (Hartzell, C. R., unpublished). It is therefore possible that the unusually strong rhombic high-spin signals observed on reoxidation with ferricyanide have a contribution from ferric cytochrome a_3.

We must also point out that the analysis given thus far has ignored complications which must be considered according to recent findings on related problems [31–34]. As our tools of observation are sharpened it becomes more and more apparent that the materials we investigate are not homogeneous in every respect. Although they may appear homogeneous by criteria of protein chemistry, they may still show heterogeneity of their active sites. If different signals, which we observe with the oxidase, should originate from different subpopulations of the functional units, and if these populations constituted sizeable fractions, our analysis could be at fault. To safeguard against such difficulties, a quantitative evaluation of the EPR signals is absolutely necessary. It is for these considerations, for instance, that we have neglected the minor low-spin component ($g = 2.6$; 2.2; 1.87). Furthermore, in the case of the concentrated preparations that we are forced to use for considerations of sensitivity, aggregation phenomena may interfere. The only criterion by which we can judge the seriousness of such complications at the moment is the behavior of the enzyme in particles. The EPR signals in particles (ETP) are indeed very similar to those seen in the purified enzyme [1, 21]. It is particularly important that the signals at $g = 6$ show similar behavior, i.e. appearance on partial reduction and only minor rhombic signals on rapid reduction (amounting to less than one half the integrated intensity of the axial signals) except on reoxidation or in the presence of added electron carriers [21]. Although the observed kinetic inhibition between the components of the oxidase on the substrate and those on the oxygen end may indeed be an artifactual feature of the purified enzyme [34], the gross features of the behavior of the EPR signals, particularly the most controversial ones, namely the high-spin signals, do not appear to deviate from those seen with the purified enzyme. However, this is clearly an area where more experimental work is required.

ACKNOWLEDGEMENTS

We are indebted to Mr W. D. Hamilton for assistance in the operation and maintenance of our instruments. This work was supported by the Institute of General

Medical Sciences, National Institutes of Health, U.S.P.H.S. by a research grant (GM-12394) and a Research Career Award (5-K06-GM-18442) to H.B.; C.R.H. is an Established Investigator of the American Heart Association.

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