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ON THE IDENTITY OF THE HIGH SPIN HEME COMPONENTS OF CYTOCHROME *c* OXIDASE

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SUMMARY

In oxidized, resting cytochrome *c* oxidase (EC 1.9.3.1) and under most conditions of partial reduction $\leqslant 50\,\%$ of the heme components are detected by EPR spectroscopy. When the enzyme is fully reduced in the presence of equimolar quantities of cytochrome *c*, anaerobic reoxidation by an excess of a chemical oxidant (ferricyanide, porphyrexide) produces intense high and low spin heme signals simultaneously. The time range in which maximal high spin signals are observed is 0.1–2 s after mixing. Under these conditions 35–50 % of the total heme *a* is accounted for by the low spin heme signal and 35–40 % by the high spin signals, with the rhombic component accounting for 30–35 % of the total heme. It is concluded that under these conditions, the major portion of both heme components must be EPR detectable. Thus, if the generally accepted assignment of the low spin signal to cytochrome *a* is adopted, it follows that in the experiments described, cytochrome a_3 is represented in the rhombic high spin signal. The quantities of heme represented in the axial high spin signal are too small for a definitive assignment; these signals could originate from either heme. When after formation of high spin signals as described, O_2 is admitted, the rhombic signal is eliminated within 4 ms. In the presence of the strongest rhombic high spin signals, the absorption band at 655 nm is only $\leqslant 25\,\%$ developed. The implications of these findings are discussed in the context of present hypotheses concerning the state and interactions of cytochrome *c* oxidase components during oxidation-reduction.

INTRODUCTION

In cytochrome *c* oxidase (EC 1.9.3.1), as it is isolated, only 50 % of the total heme is detectable by EPR spectroscopy in a low spin form ($g = 3; 2.1; 1.5$) [1, 2]. At states of partial reduction, this signal is usually diminished and, depending on conditions, one or more of a variety of high spin heme signals appear [3]. Although there seems to be general agreement now that the low spin heme signal originates from cytochrome *a*, [4, 5], the identity of the components(s) giving rise to the high spin signal(s) is uncertain. The assignment of the low spin signal to cytochrome *a* is mainly

based on kinetic data and the inertness toward ligands, as expected of a low spin heme and in fact shown by cytochrome *a* [4, 6]. Similarly it has been argued that cytochrome a_3, having high affinity for a number of ligands may be expected to be in the high spin configuration. It was largely on this basis that van Gelder and Beinert [7] attributed the high spin signals to the cytochrome a_3 component. The opposite assignment was proposed by Wilson and Leigh [8] mainly on the basis of considerations of oxidation-reduction potentials. However, as pointed out by Malmström [4] and by Nicholls [9] and Nicholls and Petersen [10], these were not compelling arguments. Leigh and Wilson [11] and Leigh et al. [12] also concluded that in the presence of CO, cytochrome *a* appears in a low spin form with *g* values practically identical to those of the supposed low spin form of cytochrome a_3. Hartzell et al. [6] then suggested the possibility that cytochrome *a*, originally represented in the low spin signal, may be converted into one of the major EPR-detectable high spin forms on initial reduction by substrate and subsequent intramolecular electron transfer to other components in the oxidase. This suggestion was mainly based on the observation that during reductive titrations, the low spin signal disappearance paralleled the appearance of high spin signals, whereas at early times during rapid reduction, very little high spin heme was observed. In addition, the EPR-detectable heme, low and high spin signals together, had never been found to significantly exceed 50 % of the total heme present and could thus be due to only one of the hemes. Thus, the question arose whether the major portion of cytochrome a_3 is ever detectable by EPR. Then, however, Wever et al. [13] reported that on photodissociation of the reduced CO compound of cytochrome a_3 in the presence of ferricyanide, the low spin heme was fully detectable, while in addition a rhombic high spin signal was generated in substantial quantity. Similar effects were evident from the work of Leigh and Wilson [11] and Leigh et al. [12]. Beinert et al. [14] reported that under special conditions, by reoxidation of reduced oxidase with ferricyanide, large low and high spin signals could be observed simultaneously accounting together for 70–80 % of the total heme. Obviously then, under conditions prevailing in these experiments, a substantial portion of both cytochromes *a* and a_3 must be observable by EPR and plausible assignments of the signals should be possible.

Since it is our opinion that the understanding of the mechanism of action of cytochrome *c* oxidase will be greatly advanced, when we come to an interpretation of the identity and behavior of the various high spin heme signals observed with this enzyme, we have attempted to maximize the effects observed in the experiments just mentioned and to put them on a reliable quantitative basis. Two developments were important in these attempts, namely the introduction of a reliable procedure for the integration of rhombic high spin signals [1] and the choice of a suitable oxidant which does not interfere with the heme signals of cytochrome *c* oxidase.

MATERIALS AND METHODS

Cytochrome *c* oxidase and cytochrome *c* were purified and anaerobic procedures, rapid freeze-quenching, low temperature optical and EPR spectroscopy and their evaluation were carried out as previously described [2, 3, 14]. Cytochrome oxidase concentrations are expressed in terms of total heme *a* present. All experiments were carried out in 10 mM sodium cacodylate buffer of pH 7.2 except for those on

photodissociation and aerobic reduction of cytochrome *c* oxidase by dithionite, in which 0.1 M potassium phosphate of pH 7.5 was used. All solutions of the enzyme contained 0.1 % (w/v) Tween 20. When required, the enzyme was reduced (cf. Table I) with NADH plus cytochrome *c*, with reduced cytochrome *c* prepared by reduction with dithionite followed by Sephadex 25 treatment, with dithionite, or with cytochrome *c* plus sodium ascorbate. Except in the experiment of Fig. 3, anaerobic conditions were maintained throughout. NADH was obtained from P-L Laboratories and ascorbate from Calbiochem. Porphyrexide [15] was a gift of Dr. C. R. Hartzell.

High spin heme signals were doubly integrated according to the procedure of Aåsa et al. [1] and Hartzell and Beinert [3]. The former procedure is the one of choice when the rhombic component in the signal predominates, while with largely axial signals the two methods agreed within ± 5 %. Ferrimyoglobin was used as a standard (cf. ref. 3). The relative contributions to the high spin signal of the rhombic and axial components were estimated according to Hartzell and Beinert [3].

For an assessment of the quantity of low spin heme present, the resonance at $g = 1.5$ was compared to that of a suitable control sample that had not been reduced or had been reoxidized by O_2. The $g = 1.5$ signals from these oxidized controls, containing the same quantity of the same enzyme preparation were considered to represent 100 % of the low spin heme (cf. refs. 1 and 2). Spectra were accumulated in a Nicolet 1020A signal averager from 4 to 16 times depending on the signal to noise ratio. These spectra of the $g = 1.5$ resonance, which has an absorption, not derivative-type shape, were then singly integrated and the areas compared [16]. At the higher levels of ferricyanide (Table I), this approach could not be used because of the slope generated by the superimposed ferricyanide signal and only approximate comparisons could be made. We estimate that the values obtained for the quantity of low spin heme in the presence of 4.5–6.5 mM ferricyanide are within ± 20 % of the correct value, whereas with porphyrexide the error is probably one half that. We estimate that the integrations of the high spin signals are of similar accuracy, whereas the comparisons of the copper signals to copper signals in the resting enzyme are the most accurate.

RESULTS

In photodissociation experiments of the reduced CO compound of cytochrome *c* oxidase in the presence of ferricyanide patterned after Wever et al. [13] we were not able to obtain high spin signals exceeding 15 % of the total heme. In addition, the quantity of low spin heme present could not be determined with satisfactory precision because of the strong superimposed ferricyanide signal. This approach seemed, therefore, unsuitable for producing decisive quantitative results on the question posed.

Although in experiments involving anaerobic reoxidation of completely reduced cytochrome *c* oxidase by ferricyanide we consistently succeeded in producing large, mainly rhombic, high spin signals accounting for up to 35 % of the total heme, the presence of ferricyanide again made it very difficult to accurately determine the amount of low spin heme simultaneously present. However, we observed in these experiments that maximal high spin signals, and particularly maximal rhombic signals, were only obtained in the presence of cytochrome *c*, with a 20–50-fold excess of ferricyanide with respect to total heme *a* and in the time range of 0.1–2 s after reaction with the oxidant. With porphyrexide as oxidant under analogous conditions, we

TABLE I

HEME *a* OBSERVED IN EPR SIGNALS AFTER ANAEROBIC REOXIDATION OF CYTOCHROME *c* OXIDASE BY FERRICYANIDE OR PHORPHYREXIDE

Nos.	Reaction time (ms)	Final concentration of reactants						High spin heme ($g = 6$)				Low spin heme ($g=1.5$) (%)	High +low spin heme (%)	Percent Cu reoxidized
		Cytochrome *c* oxidase (μM)	Cytochrome *c** (μM)	Ascorbate (μM)	NADH (μM)	Ferricyanide (mM)	Porphyrexide (mM)	Total (μM)	(%)**	Rhombic (%)	Axial (%)			
			(ferro)											
1	6	280	280	190		4.5		52	18.5	11	7.5	39	57.5	73
2	100	280	280	190		4.5		73	26	18.5	7.5	45	71	93
3	430	280	280	190		4.5		115	41	32.5	8.5	–	–	110
4	1500	280	280	190		4.5		118	42	35	7	47	89	112
			(ferri)											
5†	6	280	280	350		11		75.5	27	19	8	40	67	109
6†	1000	280	280	350		11		75.5	27	22.5	4.5	50	77	114
			(ferro)											
7	100	280	280	190		11		95	34	27	7	–	–	133
8	1000	280	280	190		11		118	42	36	6	–	–	130
			(ferro)											
9	1500	280	10		2800	6.5		103.5	37	26	11	40	77	100
			(ferri)											
10	600	300	300	400			5	105	35	30	5	42	77	–
11	1000	300	300	400			3	105	35	30.5	4.5	42	77	–

* Note that in some experiments ferri- and in some ferrocytochrome *c* was used. The ascorbate was adjusted accordingly.

** Percent of total heme *a*.

† 110 mM formate was present in this experiment.

were then able to obtain high spin signals of similar magnitude and the desired accuracy in the determination of the low spin heme. Porphyrexide, in its oxidized state, is a free radical. Because of the excess used, it, therefore, superimposes a strong radical signal on the copper resonance of cytochrome *c* oxidase. The state of the EPR detectable copper component cannot, therefore, be observed by EPR in the presence of porphyrexide, but it does not interfere with the observation of the signal at $g = 1.5$. The oxidation state of the copper was, however, monitored by low temperature reflectance spectroscopy. Porphyrexide has a weak light absorption with a broad maximum at 460 nm and negligible absorption at > 600 nm and does not seriously interfere with optical spectroscopy at the concentration used, except for imparting some background slope to the reflectance spectra. The midpoint oxidation-reduction potential of porphyrexide is given as 0.725 V at pH 7.0 (18 °C) [15]. Representative results of these experiments are summarized in Table I. The first set of four experiments shows the optimum time range in which high spin signals are found. Experiments (not shown) extending the reaction time to several seconds or minutes showed that these signals decrease again after approx. 1–2 s. It can also be seen that, while the rhombic signal grows with time, the axial signal does not change much or, if anything, decreases with time. From the quantities involved, it is obvious that the increase in detectable rhombic component does not proceed at the expense of the axial component. Thus, the notion that with time the axial component becomes converted into the rhombic one, cannot be correct, at least in the present situation. It should be recalled that it has been shown previously [14] that axial and rhombic components are not in a rapid equilibrium. Another feature to which we had drawn attention previously [14] is the

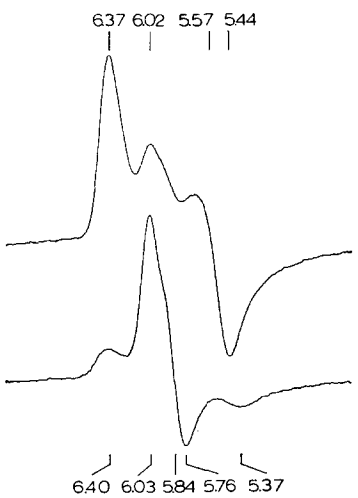

Fig. 1. EPR signals of high spin heme observed in cytochrome *c* oxidase on partial anaerobic reduction or on anaerobic reoxidation of the reduced enzyme by porphyrexide. The upper curve was recorded with Sample 11 of Table I. The lower curve was obtained with a sample of enzyme partially reduced with ferrocytochrome *c* and frozen within 1 min in liquid nitrogen. The conditions of EPR spectroscopy were: microwave power, 2.7 mW and frequency 9.22 GHz; modulation amplitude, 8 G and frequency 100 kHz; temperature, 13 K and scanning rate 200 G/min. Field positions of prominent features of the spectra are given on a scale of *g*-factors.

finding that the copper signal observed immediately after oxidation with ferricyanide is significantly larger than that of the resting enzyme.

The next two experiments (Table I, Expts. 5 and 6) show that formate does not enhance the development of high spin signals. Formate is the strongest ligand known for high spin heme, which does not lead to a conversion to the low spin state. Experiment 9 shows that the presence of cytochrome *c* in more than trace amounts is needed for optimal formation of rhombic signals. In our attempts to arrive at assignments of the high spin signals, it seemed to us an important point to have as much of the signal intensity as possible in one species, in the present case in the rhombic signal, so that it could be unambiguously established that the component giving rise to this signal cannot be the same as that also represented in the low spin signal. Because of the errors involved in the quantitative determinations this might have remained uncertain, if, e.g. the intensity were distributed equally between axial and rhombic components.

In experiments 10 and 11, porphyrexide was used as the oxidant. Although this compound has an oxidation-reduction midpoint potential almost 300 mV higher than ferricyanide, the general pattern of reoxidation was not different with the two oxidants. Intense rhombic signals were observed and the low spin heme simultaneously represented in the signal at $g = 1.5$ could be quantitatively determined with the desired

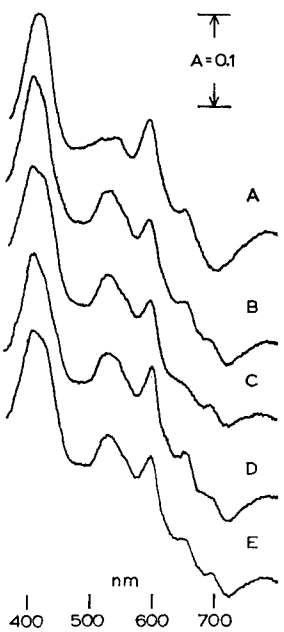

Fig. 2. Low temperature (100 K) optical reflectance spectra of cytochrome *c* oxidase reduced with cytochrome *c* and ascorbate and reoxidized with ferricyanide, in the absence and presence of formate, and corresponding control samples. The concentrations of enzyme, cytochrome *c* and ferricyanide, when added, were identical throughout. All samples were collected in the same fashion by the rapid freeze-quench procedure. A, resting cytochrome *c* oxidase without additions; B, as A, but with ferricytochrome *c*; C, as A, but reduced with ascorbate and ferrocytochrome *c* and reoxidized with ferricyanide (experiment 8, Table I); D, as B, but containing 110 mM sodium formate; E, as D, but reduced with ascorbate and reoxidized with ferricyanide analogous to experiment 6 of Table I.

accuracy (see Materials and Methods). Fig. 1 shows the signal observed in experiment 11. For comparison there is also shown a signal recorded with the same preparation when it was partially reduced with reduced cytochrome *c*. Although the properties of the rhombic and axial components observed under both of these conditions are similar, the rhombic splitting (peak to peak) in the lower spectrum is 10 % (19 G at 9.22 GHz) larger, which raises doubts about the identity of these two species.

Low temperature reflectance spectra from experiments 8 and an experiment analogous to 6 and of controls are shown in Fig. 2. Three points are noteworthy in these spectra: (a) Under conditions of reoxidation with ferricyanide, when the strongest rhombic high spin signals are observed, the absorption band at 655 nm is not restored to its intensity in the oxidized enzyme (compare C with A and B) but remains at approx. 25 % of full intensity; (b) the 655 nm absorption is significantly enhanced in the presence of formate (compare B and D or C and E), despite the fact that the differences between the high spin signals in the presence and absence of formate are only minor; (c) although, after rexoidation with ferricyanide, the Cu signals are restored and actually exceed those in the oxidized controls, the absorption band at 830 nm apparently does not revert to its original state. We have no suggestion to explain this last observation but will discuss the other points below.

Since several types of high spin signals have been observed with cytochrome *c* oxidase, which are mostly found superimposed on each other in experimental spectra, it is of interest to obtain records of the individual types, with minimal interference by others and by signals from the other components of the enzyme. To our knowledge no such spectra have been shown for cytochrome *c* oxidase. Fig. 3 shows a fairly clean spectrum of a nearly axial species obtained on aerobic reduction by dithionite. This signal, however, did not account for more than 10 % of the total heme. At the low field end of the spectrum a broad signal can be seen that has been repeatedly observed

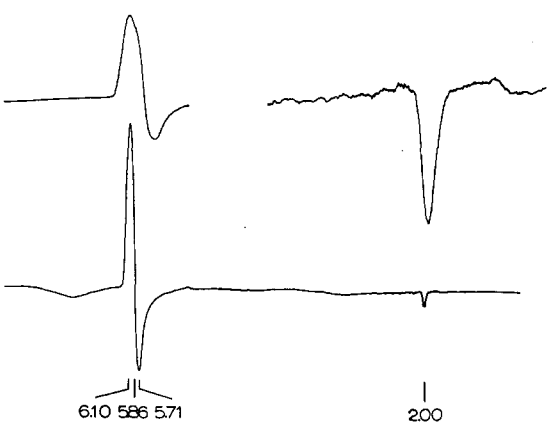

Fig. 3. Complete EPR spectrum of one of the high spin species observed with cytochrome *c* oxidase. Enzyme, 0.56 mM, in 0.1 M potassium phosphate of pH 7.5 was reduced aerobically with an excess of solid dithionite, shaken for 30 s and then frozen within 1 min in liquid nitrogen. The conditions of EPR spectroscopy for the complete curve were those of Fig. 1, except that the power was 9 mW. The partial spectra were expanded 5 (left) and 10 (right) times, respectively, along the horizontal axis and 0.5 (left) and 7.8 (right) times along the vertical axis.

by us and other investigators (cf. ref. 17). The significance or identity of this signal is not known, but it is typical for the oxidized state.

DISCUSSION

The results presented in Table I show that approx. 80 % of the total heme of cytochrome *c* oxidase can be detected, with 70–100 % of one heme represented by the low spin and 60–70 % of the other by the rhombic high spin signal, with another 8–20 % of one heme being present in the axial high spin signal. We consider the numbers of Table I as being safely beyond the limit of 50 % recovery for total detectable heme which would be expected if one component, e.g. cytochrome a_3, were completely undetectable by EPR. From the quantities involved it is also obvious that one of the hemes is represented by the rhombic high spin signal and the other by the low spin signal ($g = 3; 2.1; 1.5$), while the quantity of axial high spin signal is sufficiently low that it might originate from either heme.

The identity of cytochrome *a* or a_3 with the components giving rise to the low and high spin signals cannot be established from information available through the experiments reported here. There seems to be no reason, however, to abandon the now generally accepted assignment of the low spin signal to cytochrome *a* [4, 5], the species inert toward most ligands. This would mean that the rhombic high spin signal observed in the experiments of Table I must be attributed to cytochrome a_3. This then leads to the question as to whether all rhombic high spin signals observed with cytochrome *c* oxidase are to be attributed to cytochrome a_3. This question may never find a completely satisfactory answer for those high spin signals that represent only minor quantities of heme. Whereas the location of the three distinct *g* values and the line shapes of low spin heme compounds are very sensitive to minor changes in the environment of the iron [18, 19] so that completely identical values and spectral appearance for different compounds are practically not found, there is much less variation possible with high spin signals, the degree of rhombic splitting being one of the most useful differentiating characteristics. As shown in Fig. 1 and ref. 3, there can be differences between the size of the rhombic splitting of high spin signals of cytochrome *c* oxidase observed under different conditions. However, as in the case of Fig. 1, these differences are usually not large. The results of our experiments therefore suggest that one must consider that rhombic high spin signals observed under a number of conditions are due to the cytochrome a_3 component. We certainly favor the view that the rhombic high spin signals arising on photodissociation of the reduced CO complex [11–13] of cytochrome *c* oxidase are due to cytochrome a_3. Unfortunately, nothing definitive can be said about the axial high spin signals, of which there also are a variety [3]. Their quantity is too low and they have never been seen in the presence of (nearly) fully developed low spin signals, so that the possibility cannot be excluded that they might represent cytochrome *a* in a high spin form. It had previously been suggested by Wilson and Leigh [8] that the high spin signals represent cytochrome *a* and Hartzell et al. [6] also suggested that at least one of the types, axial or rhombic, is due to cytochrome *a*. Mechanistic schemes proposed by Nicholls [20] and Nicholls and Petersen [10] and more recently by Wikström et al. [21] imply the coexistence of cytochromes *a* and a_3 in high spin forms at approx. 50 % reduction of the oxidase, when an equilibrium between cytochromes $a^{3+}a_3{}^{2+}$ and $a^{2+}a_3{}^{3+}$ is thought to exist. One of these

species might then be represented by the axial and the other by the rhombic high spin signal (cf. ref. 10). The quantities of EPR-detectable high spin signals under most of these conditions are, however, too low so that decisive answers on these questions may have to come from measurements by techniques other than EPR by which both heme components can be detected, such as e.g. Mössbauer spectroscopy.

The experiments carried out in the presence of formate show that this ligand has no major effect, if any, on the amount of high spin heme observed by EPR when the reduced oxidase is reoxidized by ferricyanide. We have made similar observations during rapid reduction of the oxidized enzyme by ferrocytochrome *c*.

We have previously drawn attention to the behavior of the broad absorption band centered around 655 nm [3, 6, 14]. This band had been observed to show a behavior reciprocal to that of the rhombic high spin signals. This is confirmed in the present work. When the strongest rhombic high spin signals were observed, the 655 nm band was at best approx. 25 % developed. An absorption band in this region of the spectrum may be thought to be due to a high spin heme or to copper. Since formate, which is known to be a ligand for the high spin heme of cytochrome oxidase and to have an effect on its spectrum [22], increases the intensity of this band* (cf. Fig. 2) it is likely that this band is due to heme rather than copper. However, since it is not correspondingly developed when one of the hemes (a_3) is 70–80 % EPR detectable in a high spin state, the 655 nm band must be due to a high spin heme in a different state. This different state, obviously, is the EPR-undetectable one. As in previous discussions of the EPR behavior of cytochrome *c* oxidase, the question emerges: why is cytochrome a_3 undetectable in the resting enzyme or the enzyme reoxidized by O_2? and why, as we have shown here, is it detectable after reoxidation by other oxidants? Is it that these oxidants are not able to oxidize the copper component thought to be associated with cytochrome a_3, and thus prevent its interaction with cytochrome a_3? In view of this last question it was of considerable interest to us that porphyrexide, with a midpoint potential of +725 mV was unable to return the reduced oxidase into its oxidized, resting form. It seems then that it may not be a matter of oxidizing power of the oxidant but of the specificity of oxygen. In experiments in which the maximal rhombic signal was first generated with ferricyanide and thereafter O_2 was mixed in from a third syringe, the total high spin signal declined within 4 ms to represent only 3.5 % of the total heme, with 2 % of this being rhombic. Interestingly, however, the 655 nm absorption had not returned at this time. Thus disappearance of the rhombic signal on aerobic oxidation does not seem to be due to the same events that lead to restoration of the 655 nm absorption, i.e. there must be at least one intermediate state in which cytochrome a_3 has become undetectable but the 655 nm absorption is not yet developed. These aspects will be further explored.

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* This has been observed independently by Palmer, G., Iniguez-Garcia, L., Vickery, L. E., and Babcock, G. P.

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