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RESPONSES OF THE a_3 COMPONENT OF CYTOCHROME *c* OXIDASE TO SUBSTRATE AND LIGAND ADDITION

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Summary

We have previously described a transient high spin ferric heme species in cytochrome *c* oxidase (EC 1.9.3.1) which represents a_3^{3+} (Beinert, H. and Shaw, R.W. (1977) Biochim. Biophys. Acta 462, 121—130), and can be detected and quantitatively determined by EPR. We have now used our ability to generate this species to study reactions of a_3^{3+} with substrates and ligands and also responses to pH changes. This was accomplished by multiple rapid mixing and freezing techniques in conjunction with low temperature EPR and optical reflectance spectroscopies. The substrates used were O_2 and ferrocytochrome *c* and the ligands cyanide, sulfide, azide and carbon monoxide. Contrary to the oxidized, resting form of the enzyme, the transient high spin species of a_3^{3+} reacts within <10 ms stoichiometrically with cyanide and sulfide and at a slower rate with azide. The transient a_3^{3+} species responds to O_2 and CO by changes in signal size or shape, although no oxidoreduction is involved, indicating that a_3^{3+} registers the presence of these gases. The high spin signal of the transient species is readily abolished by ferrocytochrome *c* or on raising the pH. Decreasing the pH induces a shift from the rhombic towards the axial component of the signal. Since the responses to CO and pH are analogous for the rhombic transient species to those observed with the rhombic high spin ferric heme species produced on partial reduction, it is suggested that the rhombic signals represent a_3^{3+} in either case. In all these experiments, in which EPR detectable a_3^{3+} was observed in large yield, no extra signals for copper or correspondingly increased intensity in the copper signal at $g = 2$ were seen. The relationship is discussed of the obviously reactive transient species of a_3^{3+} to other 'activated' species that have been reported and to the oxidized resting form of the enzyme, which is known to react only slowly with ligands and to respond sluggishly to substrate.

Introduction

It is generally thought that cytochrome a_3, one of the two heme components of cytochrome *c* oxidase (EC 1.9.3.1), is a high spin heme compound and as such is readily susceptible to attack by a number of ligands [1,2]. They are, however, experimental problems which have thus far made it difficult to come to unambigous conclusions on the basis of optical or EPR features arising in ligand or substrate interaction of the components of the enzyme. The main difficulties are that the optical absorptions of the components overlap extensively, that absorptivities for the individual components are not known, that in the resting enzyme, whether oxidized or reduced, the a_3 component, is not detectable by EPR, and that the a_3^{3+} component in the resting enzyme does, in fact, not react with most ligands, that is, partial reduction is required before reaction is observed.

Recently we have been able, through anaerobic oxidation of completely ($4e^-$ per aa_3) reduced cytochrome *c* oxidase by chemical agents, to produce a form of the oxidase of a few seconds lifetime, in which up to 80% of the a_3 component is detectable by EPR in a rhombic high spin signal [3]. In view of the uncertainties concerning reactions of a_3^{3+} referrred to above, we have now exploited our ability to generate an easily EPR detectable and quantitatively measurable form of a_3^{3+}, by exposing this transient species to various ligands and substrates. This was accomplished by the use of multiple rapid mixing and freeze-quenching techniques so that the detectable transient form of a_3^{3+} was made to react at the optimum time of its development. The substrates or ligands used were: O_2, ferrocytochrome *c*, cyanide, sulfide, azide, and carbon monoxide. In addition, some effects of rapid pH changes were studied. Some of these experiments were reported in preliminary form [4].

Materials and Methods

These were as described or cited in our previous publication [3]. The enzyme used was prepared as in ref. 5 and 10 mM cacodylate of pH 7.4 was used as buffer unless specificed otherwise. Porphyrexide was purchased from K and K Laboratories. Solutions of potassium cyanide were evacuated and equilibrated with argon before neutralization. According to van Buuren [6], solid citric acid was placed in a side arm of the evacuation vessel. After equilibration with argon the critic acid was dissolved in the cyanide solution. As in our previous publications, concentrations of cytochrome *c* oxidase are given in terms of total heme *a* present, i.e., not distinguishing between *a* and a_3. The syringes of the rapid mixing apparatus were controlled by an Update Instrument Inc. (Madison, Wisc.) Precision Ram System. All experiments were carried out with the syringes kept at 13—15° C. For quantitative determinations, the low field peaks of the various low spin signals observed were averaged by a Nicolet 1020 signal averager. The procedures proposed by Aåsa and Vänngård [7] and by Aåsa et al. [8] were used for quantitative evaluation of the signals.

Results

Generation of EPR detectable a_3^{3+}

In our previous experiments [3], we had mainly used ferricyanide as oxidant

and an amount of cytochrome *c* approximately stoichiometric with the total heme of the oxidase. Although the evaluation of the EPR signal of a_3^{3+} that can be generated in this way is not interferred with by these additions, the excess of ferricyanide makes it difficult to impossible to arrive at more than a qualitative estimate of the *a* component, whether measured from its low or high field line. Also, low spin heme resonances from a_3^{3+}-sulfide and azide, which occur at $g \approx 2.5$—3.0 cannot be measured or even detected. These difficulties are aggravated by the presence of large quantities of ferricytochrome *c*, particularly in the evaluation of the optical spectra. In attempts to improve these conditions without sacrificing much of the yield of the intermediate, we arrived at the following more suitable conditions: 0.56 mM enzyme was reduced anaerobically in the presence of 1.2 mM ferrocyanide and 15.9 μM ferricytochrome *c* by 0.83 mM ascorbate. Porphyrexide, 10 mM, was used as oxidant. The use of porphyrexide makes it impossible to monitor the Cu signal at $g = 2$ *, however an estimate of this signal can be made from the $g_{\|}$ region, which is not interferred with. There appears also a small half-field signal at $g = 4$ from radical-radical interaction of porphyrexide. The optimum time for development of the high spin signal of a_3^{3+} was found to be between 1 and 4 s. The oxidation state of the transient form is not known with certainty. The detectable components, viz. a_3^{3+}, a^{3+} and the detectable copper are >80% oxidized but no information is available on the state of the undetctable Cu.

Reaction with O_2

Although one might a priori not expect a reaction between an oxidized form of cytochrome *c* oxidase and O_2, the transient detectable high spin species disappears in the presence of O_2 within the resolution of our mixing-quenching technique. Measurement of the reflectance spectra of the samples obtained in such experiments showed, however, that the resting form of the enzyme was not restored immediately, since the 655 nm absorption typical for this form only appeared slowly in the course of seconds to minutes [4]. Thus, there is at least one more transient form in which a_3^{3+} is undetectable by EPR but the conformation of the resting enzyme is nevertheless not restored. This might have to do with the oxidation state of the undetectable Cu or with the necessity for a conformational rearrangement involving this Cu and a_3^{3+}.

Reaction with ferrocytochrome c

The reactivity of the transient high spin species with ferrocytochrome *c* was of prime importance, particularly in view of the discovery of thus far poorly defined 'reactive' forms of the enzyme [9—11]. It may also be recalled that we had previously only seen negligible amounts of rhombic high spin species appear on rapid reduction of oxidase by ferrocytochrome *c* [12], so that the role of the rhombic high spin form in the catalytic reaction was not apparent.

In order to study reduction of the transient species by ferrocytochrome *c*, any excess of the chemical oxidant used to generate this species had to be

* Although there is some discussion whether this signal is indeed due to Cu^{2+} or to some other component interacting with Cu, for purposes of this paper, we shall call this signal the Cu signal as is generally done.

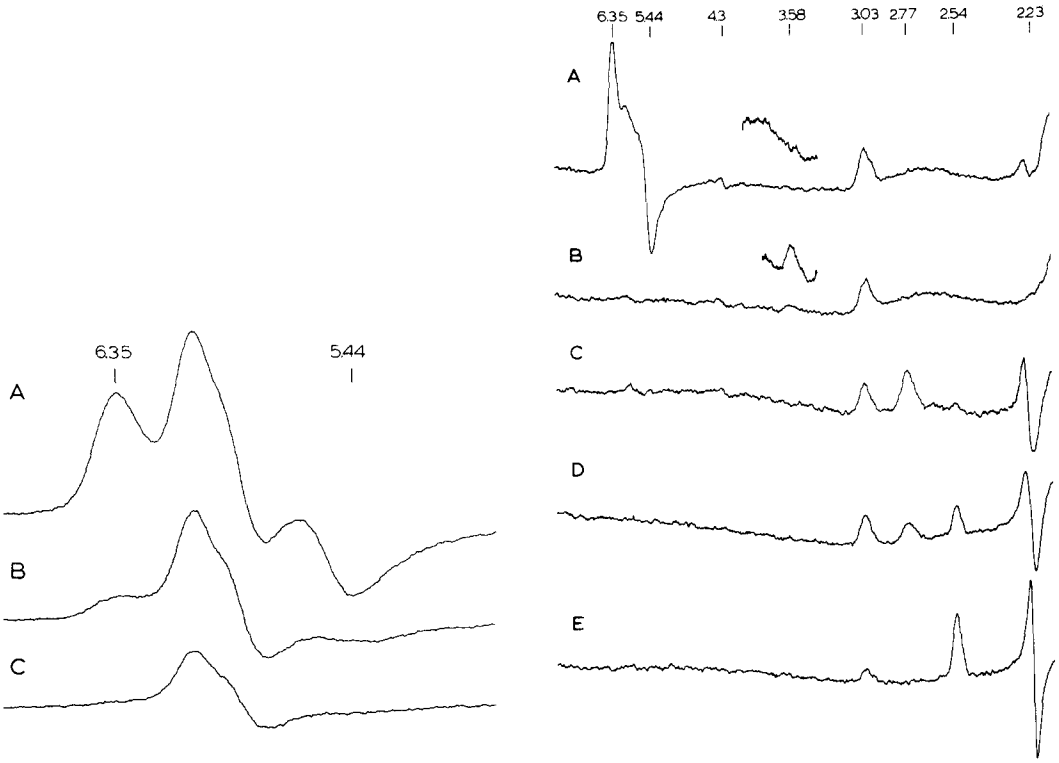


Fig. 1. (A) EPR spectra of high spin components at $g = 6$ in cytochrome *c* oxidase after generation of the transient high spin intermediate and removal of excess oxidant; (B) 10 ms after exposure of the enzyme in the state shown in (A) to ferrocytochrome *c*; and (C) enzyme, as reduced for the production of the sample shown in (A), exposed to buffer saturated with O_2 at 18°C. Because of the number of consecutive mixing operations used, the ratio of enzyme concentrations in the samples shown in A, B and C is 1.25 : 1 : 1.75. The conditions of EPR spectroscopy were: microwave power and frequency, respectively, 2.7 mW and 9.2 GHz; modulation frequency and amplitude, respectively, 100 kHz and 8 G; scanning rate 200 G/min; and temperature 13 K. Cytochrome *c* oxidase, 0.98 mM, was made anaerobic in a tonometer and then reduced with 3 mM NADH in the presence of 6 μM cytochrome *c*. The solution was drawn into a syringe and left for 42 h at 2°C. This solution was mixed at 13°C in the first step (not shown) with an equal volume of 7.6 mM porphyrexide. For the second step, the combined solutions were mixed (at a 4 : 1 ratio) after 1.5 s with a 7 mM solution of NADH in 25 μM Tris-chloride of pH 8 and frozen after 100 ms (A). The yield of the transient high spin species was 41% of 1 heme at this point. In the third step the mixture obtained in the second step was not frozen but further mixed with a solution of 2 mM ferrocytochrome *c* (at a 2.5 : 1 ratio) and frozen after 8 ms.

Fig. 2. EPR spectra of cytochrome *c* oxidase recorded in experiments in which the transient high spin species was exposed to cyanide or sulfide. A, enzyme in the transient state as produced by oxidation with porphyrexide at 820 ms and then mixed with anaerobic buffer (at a 2 : 1 ratio) for 6 ms; B, as A but mixed anaerobically with a 50 mM solution of KCN (see Methods) instead of buffer; C, D and E, as in A but mixed anaerobically with a 40 mM solution of NaHS (pH 7.4) for 6, 200 and 6600 ms, respectively. The final enzyme concentrations were 205 μM in A—C and 192 μM in D and E. The conditions of EPR spectroscopy were as for Fig. 1 except that the scanning rate was 500 G/min. For the inserts in A and B, an amplification was used 5-fold higher than for the main spectra and the time constant and scanning time were increased.

removed. An excess of oxidant is however required for optimum formation of the intermediate. We, therefore, reduced porphyrexide, after generation of the transient, by an excess of NADH. This reaction occurs in 10 ms. NADH,

on the other hand, reduces the oxidase only slowly in the course of hours. Ferrocytochrome *c* was then introduced from a fourth syringe. The rhombic high spin signal disappeared within a few milliseconds (Fig. 1).

Reaction with cyanide

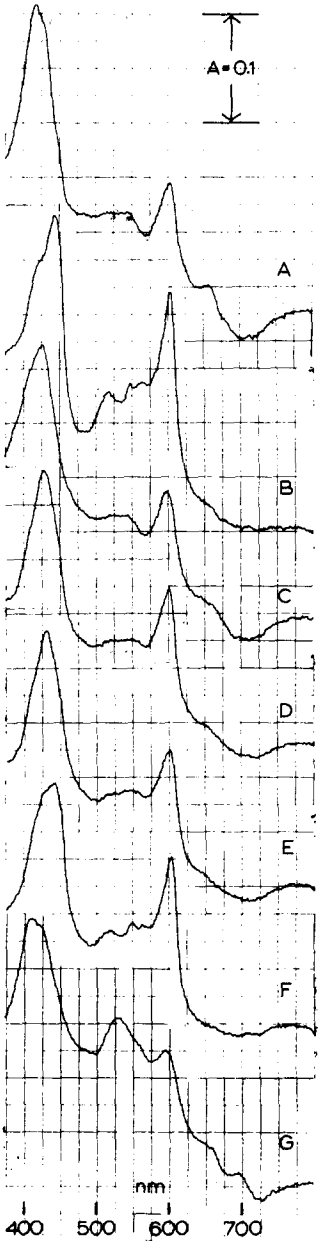
Although reaction of the transient high spin species with cyanide was expected, the rate of the conversion and its quantitative aspects deserved exploration. In the presence of 17 mM KCN, the high spin species disappeared within the resolution of our apparatus (Fig. 2) and the low field resonance of the low spin a_3^{3+} cyanide appeared ($g = 3.58$). Within the accuracy of determinations, the conversion was complete (cf. Table I).

Reaction with sulfide

Sulfide as well as cyanide and azide react rapidly only with partially reduced forms of the enzyme. Thus, the typical EPR signals of a_3^{3+} sulfide are generally only seen in partially reduced states of the enzyme. Sulfide itself, of course, will lead to partial reduction. The transient high spin species generated by anaerobic reoxidation reacted instantly with 13 mM sulfide as shown in Fig. 2. It is also apparent from this experiment that the form of a_3^{3+}-S^{2-} described in the literature [13] with *g* values at 2.54, 2.23, 1.87 is not the first product of the reaction of the transient species with sulfide (Fig. 2C), but a species with $g = 2.77$, 2.23, 1.71. Within a few seconds this is then completely converted to the species previously described (Fig. 2D and E). As in the case of cyanide, the conversion of the high spin transient to the low spin sulfide derivative was complete. Optical reflectance spectra from this experiment are shown in Fig. 3. It is apparent from the optical as well as the EPR spectra that there is increasing reduction of most components with increase in exposure time to sulfide. It is possible that the 'late' a_3^{3+}-sulfide species is typical for enzyme units in which *a* is reduced. Following the report of Seiter et al. [14] on the appearance of a special signal for the undetectable copper after formation of a_3^{3+} sulfide, we carried out a number of experiments mixing sulfide either with resting cytochrome *c* oxidase or with the transient high spin form generated with ferricyanide or porphyrexide as oxidant in the time range from milliseconds to minutes. Ferricyanide was used in addition to porphyrexide because of the interference of the radical signal from porphyrexide with the $g_\perp$ region of the Cu signal. Although a_3^{3+} sulfide was formed as usual, we failed to find, even at 6 ms reaction time, any significant new signal or increase in the copper signal.

Reaction with azide

It has been reported by Wever et al. [13], that in the dark the strength of ligands for a_3^{3+} has the order $N_3^- < S^{2-} \simeq CN^- < CO$. As shown in Fig. 4, the reaction of 50 mM azide with the transient high spin species requires several hundred ms for completion. The a_3^{3+} azide species formed ($g = 2.77$, 2.2, 1.76) rapidly, as in the case of sulfide, is not the generally observed major azide species ($g = 2.9$, 2.2, 1.67). However, the $g = 2.77$, 2.2, 1.76 species has also been seen previously in experiments carried out on a slower time scale [3,13, 15,16]. Since no significant changes are observed in the intensity of the low spin signal at $g = 3.0$, ascribed to the *a* component, we conclude from these



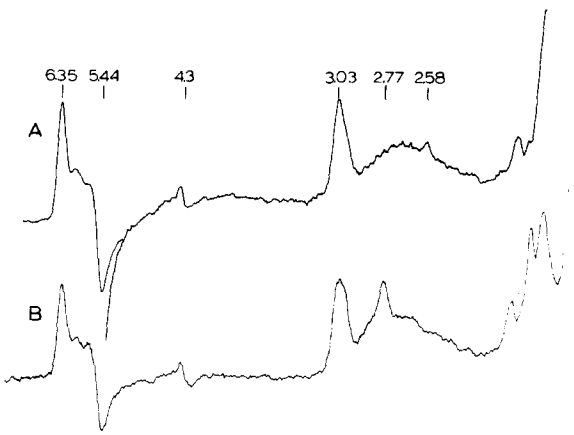


Fig. 3. Optical reflectance spectra recorded at 100 K of samples from the experiments of Fig. 2 and of control samples. A, oxidized resting enzyme; B, reduced enzyme mixed with anaerobic buffer; C, enzyme after generation of the transient high spin species (sample of Fig. 2A); D, E and F, as C but mixed with sulfide (samples of Fig. 2C, D and E); G, sample reduced by ascorbate in the presence of an equimolar amount of ferricytochrome *c* (cf. Fig. 2), reoxidized anaerobically by porphyrexide and then exposed to CO for 3 min before freezing.

Fig. 4. EPR spectra of cytochrome *c* oxidase recorded in experiments in which the transient high spin species was exposed to azide. A, enzyme in the transient state at 1.4 s and then mixed with anaerobic buffer (at a 2 : 1 ratio) for 6 ms. B, as A but mixed anaerobically with a 150 mM solution of NaN_3 for 200 ms instead of buffer. At 500 ms, the high spin signal had completely disappeared. The final enzyme concentration was 215 μM in both samples. The conditions of EPR spectroscopy were those of Fig. 2. The insert (signal at $g = 6$) in A only was recorded at an amplification 0.39 times that of the main spectra.

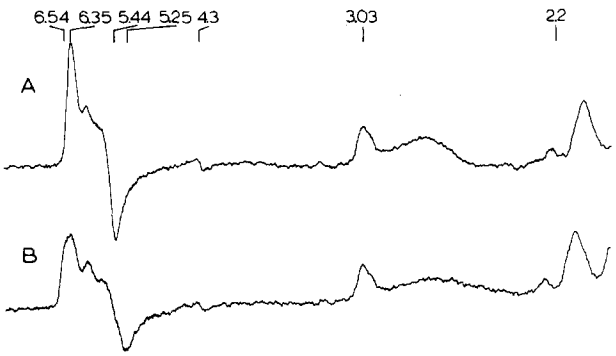


Fig. 5. EPR spectra of cytochrome *c* oxidase recorded in experiments in which the transient high spin species was exposed to CO. A, enzyme in the transient state at 1.4 s and then mixed with anaerobic buffer (at a 2 : 1 ratio) for 6 ms. B, as A but mixed anaerobically with a 1 mM solution of CO. The final enzyme concentration was 191 μM and the conditions of EPR spectroscopy were those of Fig. 2. At 100 ms, the signal at $g = 6$ represented only the form of high rhombicity (with peaks at $g = 6.56$ and 5.25, cf. Fig. 4).

experiments that under these conditions at least, azide reacts with a_3^{3+} and not with *a* as had been suggested [17,18].

In the experiments with addition of azide we made another observation bearing on the identity of the minor low spin species which is sometimes observed at low concentration in samples at pH 7.4 and which is more generally seen at more elevated pH (see below). A small signal of this species was present in the sample to which azide was added. This signal disappeared on addition of azide together with the high spin signal at $g = 6$. This lends support to the idea that the minor low spin species originates from a fraction of the a_3 component [19,20], as had been suggested from similar observations involving carbon monoxide and nitric oxide as ligands.

Reaction with carbon monoxide

As shown in the epxeriment of Fig. 5, the transient high spin species, representing a_3^{3+}, immediately (6 ms) responds to the presence of CO by an increase in rhombicity of the EPR signal. The features found at $g = 6.35$ and 5.44 in the transient species before addition of CO are found at $g = 6.54$ and 5.25 after addition. Although the signal decreases in amplitude, the total integrated intensity represented in the signal remains unchanged (cf. Table I). While at 6 ms, as shown in Fig. 5, both species, the original transient and the reaction product with CO, are present, at 100 ms the original unreacted form had disappeared (not shown). At later times (30 s) the whole signal disappeared completely.

Responses to pH changes

As shown in previous work [16], the intensity and shape of the high spin ferric heme signals of cytochrome *c* oxidase observed at states of partial reduction with cytochrome *c* depend on pH. At pH ≈ 9 no high spin signals appear, at pH ≈ 7.5 a mixture of axial and rhombic species is observed, while at pH ≈ 6 relatively intense axial signals appear, broader than those seen at neutral

pH. It was, therefore, of interest to expose the transient high spin species we have described [3] to changes in pH. The transient species, generated in the usual way, was subjected to a pH jump by mixing anaerobically with buffer of a suitable pH, so that there should be no change in the overall oxidation-reduction level. Similar experiments had previously been carried out on enzyme partly reduced with ferrocytochrome *c* [12]. A very rapid (<6 ms) equilibration between axial and rhombic components had been observed when the pH was changed from 7.5 to 6, with the axial signal increasing and rhombic decreasing. Analogous observations were made in the present work with the transient species, again reconfirming the identical behavior of the rhombic high spin species generated as a transient or obtained at equilibrium during titration. When a jump to pH 9.2 was carried out with the transient species, all high spin signals disappeared within 25 ms, the earliest observation time. It was of particular interest in these experiments whether signals of the minor low spin species of the oxidase (g = 2.6, 2.2, 1.8; refs. 15, 16, 19, 20) would appear concomitant with the disappearance of the high spin signals. This minor low spin species has generally been observed at states of partial reduction at pH > 7.5 and it had been suggested that it is related to the high spin species appearing during partial reduction at neutral pH. A quantitative relationship had, however, never been demonstrated, i.e., appearance of the intensity in the low spin signal which disappeared from the high spin signal. When the transient high spin species was exposed to a jump from pH 7.2 to 9.2, the low spin signals were no stronger at 25 ms than they were in the control sample at pH 7.2, while the high spin signals had disappeared completely. However, after 3 min at pH 9.2 (Fig. 6), the low spin signal increased significantly. The quantitative relationships are shown in Table I. More precisely, the species present initially (g = 2.59) largely disappeared and a species at g = 2.58 appeared. Obviously, the low spin signal falls far short from accounting for the intensity lost with the high spin signal. Similar results were obtained when the pH jump was carried out before the reoxidation of the reduced enzyme by

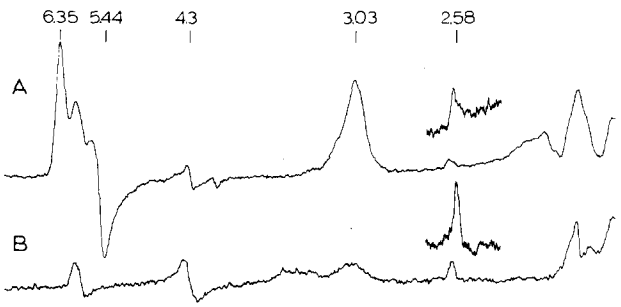


Fig. 6. EPR spectra of cytochrome *c* oxidase recorded in experiments in which the pH of the transient high spin species was rapidly adjusted to 9.2. A, enzyme in the transient state at 1.2 s; B, mixture as generated for A, mixed with 0.2 M glycine (pH 10.6) and frozen after 3 min. The final enzyme concentration was 328 μM in A and 219 μM in B. The conditions of EPR spectroscopy were those of Fig. 2. The inserts at g = 2.6 are recorded at an amplification 3.2-fold higher than that used for the main spectra and the time constant and scanning time were increased. Note that there is a low spin species present at $g \doteq 2.59$ in A. A different species (g = 2.58) arises in B.

TABLE I

CONVERSION OF TRANSIENT HIGH SPIN SPECIES (a_3^{3+}) INTO PRODUCTS ON REACTION WITH LIGANDS AND ON CHANGE OF pH

Addition (mM) *	Reaction time (ms)	EPR intensity from resonance at *g-value* listed (% of 1 heme ** in aa_3)				Conversion of transient to reaction products (% of intensity at g = 6.6—5.2 in control)	EPR intensities of heme species combined (% of total heme in aa_3)
		g = 6.6—5.2	3.58		3.0		
None		50			85	0	68
KCN, 17	6	0	51		74	102	62
		g = 6.6—5.2	2.77	2.54	3.0		
None		50			85	0	68
NaHS, 13	6	0	67	7	70	148 ***	72
None		45			93	0	69
NaHS, 13	200	0	29	23	74	114 ***	62
NaHS, 13	6600	0	0	54	32	120 ***	43
		g = 6.6—5.2	2.93	2.77	3.0		
None		51			84	0	67
NaN_3, 50	200	15	4	17	80	40	58
None		49			86	0	67
CO, 1	6	50			82	102	66
	min	g = 6.6—5.2	2.59	2.58			
None		64	~5	~3	—	0	—
Glycine pH 10.6, 67	3	<1	~1	13	—	20	—

* All values for concentrations refer to the final concentrations after completion of both mixing steps. The enzyme concentration varied from experiment to experiment within the limits of 192—215 μM total heme *a* present.

** The enzyme (aa_3) is assumed to contain equal amounts of cytochromes *a* and a_3 and total heme was determined from: $\Delta A^{\text{red-ox}}_{605\text{ nm}} = 12.0 \cdot \text{mM}^{-1}\ \text{cm}^{-1}$.

*** Note that sulfide, as a reductant, is able to form the low spin $a_3^{3+} - S^{2-}$ compound from the resting form $a^{3+}\,a^{3+}$. Thus the high values may be related to the fact that sulfide is able to lead to conversion of a portion of a_3^{3+} to the low spin state which was not in the EPR detectable transient form.

ferricyanide or porphyrexide. Thus, it is evident that there is at least one intermediate state involved in the transition from neutral to alkaline pH, in which neither a high spin nor the minor low spin signal is observed.

Discussion

After we had shown that a readily EPR detectable form of a_3^{3+} could be generated [3], it was a logical step to take viz. attempting to demonstrate that this species indeed shows behavior and reactions expected of a_3^{3+}. It should be recalled here that, in the oxidized resting enzyme, a_3^{3+} does not react with cyanide, sulfide or azide although it is the oxidized form of a_3, that binds to these ligands. It is known that on partial reduction of the oxidase or during catalytic turnover, these reactions occur readily. However, in this case only a fraction of a_3 can react since the rest has been reduced, unless this is reversed by addition of an oxidant. We have shown in this paper that the transient high

spin form of a_3^{3+} that can be generated on anaerobic reoxidation of completely reduced oxidase, reacts completely with cyanide and sulfide within the resolution of our technique (10 ms), forming the corresponding ligated low spin forms. As we have also shown, it responds on the same time scale to the addition of CO and to changes of pH. The reaction with azide is considerably slower, with at $t_{1/2}$ of approx. 100 ms. However, our quantitative data (cf. Table I) leave no doubt that it is a_3 that reacts with azide under these conditions and not *a*.

We will now summarize the salient points that we think have emerged from the experiments reported above.

Cyanide

The conversion of the transient high spin species of a_3^{3+} to the low spin cyanide species is very rapid and stoichiometric. The a_3^{3+}-CN species [21] which is known from equilibrium type experiments, appears to be formed.

Sulfide

The reaction with wulfide is of similar rate, but in this case a new a_3^{3+}-S^{2-} species (g = 2.77, 2.23, 1.71) is formed which only in the course of a few seconds is converted to the species known from equilibrium experiments (g = 2.54, 2.23, 1.87) [13]. Since sulfide is an efficient reductant for the enzyme, it is important to point out here that the conversion of the transient high spin species is stoichiometric (cf. Table I), i.e., that the reaction of a_3^{3+} with sulfide to form the low spin species is more rapid than its reduction by sulfide which possibly may involve *a* and/or copper. In relation to a report that during reduction by sulfide and partial formation of a_3^{3+}-S^{2-}, EPR undetectable copper becomes detectable [14], we can state on the basis of our experiments with any of the ligands used here that we have not observed a significant increase in the copper signal or new signals attributable to copper, other than the small, variable increase in intensity reported previously [3,12] which may raise the quantity of detectable copper to ≈50% of the total copper present in the enzyme.

Azide

There has been some doubt as to which of the heme components of cytochrome *c* oxidase reacts with azide [16—18]. The ligand replacement experiments of Wever et al. [13,22] certainly lent weight to the conclusion that a_3 is the component binding azide. Our experiments confirm this as well as the relatively low affinity of azide for a_3 as compared to that of cyanide or sulfide. This does not exclude that under different conditions azide may also react with *a*. As in the case of sulfide, with azide a species is formed first (g = 2.77, 2.2, 1.76) different from that mainly seen in equilibrium type experiments (g = 2.9, 2.2, 1.67) [4,13,15,16,22]; although the g = 2.77 species has been observed previously as a minor species [15,16,22]. The similarity in g values of the early sulfide and azide species makes it tempting to suggest that the heme invironment is these early species is almost identical.

Carbon monoxide

CO is generally thought to react only with the reduced oxidase, viz. a_3^{2+}.

However, there is evidence that CO cannot freely diffuse away from heme proteins and that bound intermediate states exist [23,24]. We have also shown previously that the low spin EPR spectrum of the *a* component of cytochrome *c* oxidase is sensitive to the presence of gas in solution at the time of freezing of the sample [5]. The notion of a gas channel or pocket in the protein leading to the heme prosthetic group which is capable of interacting with O_2 or CO has therefore found appeal [25,26]. Our experiments, reported above, in which it is shown that even the oxidized form of a_3 readily senses the presence of carbon monoxide, supports these ideas.

It is interesting at this point to make some relevant comparisons with the response of a_3^{3+} to carbon monoxide reported above. On reaction with O_2, the transient high spin species disappears within 6 ms and the 655 nm absorption band returns within seconds to minutes. Neither of these events occurs on reaction with CO. However, CO does lead to the disappearance of the high spin signal while there is no evidence from the reflectance spectra that any component of the enzyme is reduced (Fig. 3G), i.e., there are no reducing equivalents in the system which would favor formation of a_3^{2+}-CO. CO might thus be able to produce a form akin to that obtained on reaction of the transient species with O_2 in the first $\approx$30 s, namely one in which there is neither 655 nm absorption nor a high spin signal observed (see above). Secondly, concerning the increase in rhombicity of the high spin signal of the transient species, we may recall here the observations made on photodissociation of the partly reduced enzyme in the presence of CO and ferricyanide [27]. In this case a significant rhombic high spin signal with low and high field peaks at $g = 6.58$ and 5.25 is observed. Even before our own work, in which we were able to generate the EPR detectable high spin form of a_3^{3+} in large quantity and provide strong evidence for its identity, it had been generally assumed that the rhombic high spin species arising on photodissociation of a_3^{2+} CO in the presence of an oxidant represents a_3^{3+} [22,27].

Another point of considerable interest results from a comparison of the behavior of the transient high spin species with that observed, though at lower concentration, at equilibrium, e.g., on partial reduction with ferrocytochrome *c*. We had shown previously [12] and reconfirmed this here that, when that species is exposed to CO, there is also an immediate increase in rhombicity, of a magnitude similar to that seen in the present work, viz. from low and high field peaks at $g = 6.41$ and 5.37 to $g = 6.56$ and 5.21, respectively. From the analogous response of both species, namely the transient one described here and that generated under equilibrium conditions, one is tempted to conclude that both rhombic species represent the same component of the oxidase, namely a_3^{3+}.

Changes of pH

The experiments reported here add to the evidence that the minor low spin heme signals particularly observed at elevated pH ($g = 2.6$, 2.2, 1.8) are due to low spin forms of a_3^{3+}. This had been made very likely by the previous demonstration by Wever et al. [19,20] that this species disappears on addition of NO or CO. We have also observed the other signals ($g = 3.36$ and 3.27) described by these authors after adjustments of pH towards the alkaline range.

In the responses towards changes in pH, both towards the acid or alkaline range, we notice the same correlation between the rhombic transient high spin species and that observed on partial reduction at equilibrium which we stressed above in the discussion of the response to carbon monoxide. This adds to the evidence that both species represent a_3^{3+}.

Activated forms of cytochrome c oxidase

All the observations on the reactions of the transient a_3^{3+} with ligands, that we reported above, make it clear that a_3^{3+} in the resting, oxidized form of the enzyme is in a state which is unreactive towards these ligands. Evidence is accumulating that the resting enzyme reacts with substrate at a much lower rate than 'primed' or 'activated' forms [9—11]. It is tempting to propose that it is the same unreactive character of a_3^{3+} in the resting enzyme which is the reason for the low activity with ligands as well as with substrates and that the basis for this is a specific interaction with the EPR undetectable copper [15,28,29]. Characteristic for the resting, oxidized, and thus for the unreactive state seems to be the 655 nm absorption band. This band is absent in the transient, reactive EPR detectable high spin species. If the 655 nm absorption band indeed signals interaction of a_3^{3+} with the undetectable copper to form the unreactive resting enzyme form, it remains a puzzle, why, in the forms in which a^{3+} is reactive and EPR detectable, the undetectable copper does not also become observable. One must, therefore, seriously consider that, as voiced previously [30—33] this copper is in a state of indeterminate valency and cannot be considered simply as Cu^{2+}.

It is difficult at this point to compare the reactive transient species studied by us with 'activated' species reported by others. In many cases, the presence of such species was deduced from kinetic observations and their identity is, therefore, poorly defined. According to our experience, however, any species generated by activation with oxygen (as most 'activated' forms are) is likely to have the 655 nm absorption and should therefore differ from our transient high spin species. We may recall here, however, that we have observed in the presence of oxygen, a second transient species that has neither the rhombic high spin signal nor the 655 nm absorption. However, this species arises from an oxidized form, viz. our transient intermediate species, whereas 'activated' forms reported by others are produced on exposure of a reduced form to oxygen.

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