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OXIDO-REDUCTIVE TITRATIONS OF CYTOCHROME *c* OXIDASE FOLLOWED BY EPR SPECTROSCOPY

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SUMMARY

Experiments are described on oxido-reductive titrations of cytochrome *c* oxidase as followed by low-temperature EPR and reflectance spectroscopy. The reductants were cytochrome *c* or NADH and the oxidant ferricyanide. Experiments were conducted in the presence and absence of either cytochrome *c* or carbon monoxide, or both. An attempt is made to provide a complete quantitative balance of the changes observed in the major EPR signals. During reduction, the maximal quantity of heme represented in the high-spin ferric heme signals ($g \sim 6; 2$) is 25 % of the total heme present, and during reoxidation 30 %. With NADH reduction there is little difference between the pattern of disappearance of the low-spin ferric heme signals in the absence or presence of cytochrome *c*. The copper and high-spin heme signals, however, disappear at higher titrant concentrations in the presence of cytochrome *c* than in its absence. In these titrations, as well as in those with ferrocytochrome *c*, the quantitative balance indicates that, in addition to EPR-detectable components, EPR-undetectable components are also reduced, increasingly so at higher titrant concentrations. The quantity of EPR-undetectable components reduced appears to be inversely related to pH. A similar inverse relationship exists between pH and appearance of high-spin signals during the titration. At pH 9.3 the quantity of heme represented in the high-spin signals is < 5 %, whereas it approximately doubles from pH 7.4 to pH 6.1. In the presence of CO less of the low-spin heme and copper signals disappears for the same quantity of titrant consumed, again implying reduction of EPR undetectable components. At least one of these components is represented in a broad absorption band centered at 655 nm. The stoichiometry observed on reoxidation, particularly in the presence of CO, is not compatible with the notion that the copper signal represents 100 % of the active copper of the enzyme as a pair of interacting copper atoms.

INTRODUCTION

With the introduction into biochemistry of EPR spectroscopy, which potentially allows us to observe the metal components in proteins directly, research on

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cytochrome *c* oxidase has received new impetus. The expectation, however, that by EPR spectroscopy we may be able to recognize in a straightforward manner the four metal components of the active unit [1, 2], namely the two cytochromes *a* and a_3 and two copper atoms, was not realized. In the oxidized enzyme, as isolated, what appears to be only a single low-spin heme species and a single copper species is observed by EPR. These two species cannot even account for half the capacity of the enzyme for electron uptake. On partial reduction, however, the presence of several high-spin ferric species becomes apparent, so that the number of different EPR-detectable heme species in fact exceeds the number of heme components of the enzyme. Since even the long practised spectrophotometric methods have not led to unambiguous assignments of all light-absorption and ligand-interaction features, it is understandable that the multiplicity of components observed by EPR has only added to the complexity of the situation. The low- and high-spin heme signals have been attributed variously to cytochrome *a* or a_3, or both [1, 3–5] and it is not likely that a decision will come from EPR spectroscopy and spectrophotometry alone. Just for this reason it seems necessary that the information obtainable by EPR be laid down in quantitative terms, so that it may eventually be correlated with results from other approaches. This is what is being attempted in this series of papers [6, 7]. The first paper [6] dealt with the characteristics of the various EPR signals per se, while data on oxidation-reduction under equilibrium and non-equilibrium, initial reaction conditions, will be reported in this and the accompanying paper [7].

In the work to be summarized in this paper, the components of the enzyme were brought into a number of different states by the choice of reductant, oxidant after previous reduction, pH, and the presence of CO and azide, and whenever feasible we have attempted to obtain a quantitative balance of components reduced or oxidized.

MATERIALS AND METHODS

Materials and methods were essentially those described previously [6, 8, 9]. A number of high-spin ferric heme signals were also integrated by the new procedure worked out by Aåsa et al. [19]. The results of these integrations agreed within 10 % with those obtained by the method used here [6]. The enzyme preparation used throughout this study, except when specifically stated otherwise, was that described by Hartzell and Beinert [6]. This preparation, obtained from beef heart mitochondria by extraction with Triton X-114 and solubilization with cholate, contains $< 5\,\%$ phospholipid and ≤ 14 μmol heme/g protein. All experiments reported here were carried out anaerobically in vacuo or with ~ 1 atm. of CO present, when indicated. The CO was drawn from a reservoir in which commercial CP grade CO had been stored for several days over alkaline pyrogallol. Cytochrome *c* was reduced with sodium dithionite and passed through Sephadex G-25. Cytochrome *c* was estimated in its reduced form from absorption spectra recorded after titration of a particular sample at room temperature before freezing [10]. Reflectance spectra [11] were recorded after freezing at 100 °K to ascertain whether any significant change in the oxidation-reduction balance of the components had occurred. Cytochrome *c* was also estimated in its oxidized form from the resonance at $g = 3$. Since this resonance overlaps that of the low-spin heme of cytochrome *c* oxidase, the signal size was

measured at $g = 3.14$, at a field value, where the intensity of the cytochrome oxidase heme signal is negligible.

A few terms which we use in this and the accompanying paper [7] require definition. The enzyme concentration is expressed in terms of the molarity of total heme *a*, not differentiating between cytochromes *a* and a_3. Heme *a* was determined (cf. ref. 6) from $\Delta A_{red\text{-}ox}$ at 604 nm using a ΔA_{mM} of 12.0 [12]*. The concentration of all reactants refers to the final molarity achieved, after mixing, at the time a reaction was allowed to take place, except when it is explicitly stated that a reactant of a specific molarity was mixed with another reactant at a given molarity. Corrections were made for solvent loss in samples exposed to vacuum. As pointed out previously [5], it is not possible without additional information to equate unambiguously EPR signal appearance or disappearance with oxidation or reduction, respectively. In the conclusions drawn from this work, this reservation has to be kept in mind. The data in the figures, unless expressed simply as percent of maximal signal, are expressed in terms of molarity of a component present per molarity of total heme *a*. On the basis of our work [6], we assume for this kind of evaluation that the copper signal in the enzyme, as isolated at pH 7.4, represents 0.35 mol of copper per mol heme *a* present. The low-spin heme ($g = 3$; 2.2; 1.5), however, is now taken to represent 50 % of the total heme of cytochrome *c* oxidase. Aåsa and Vänngård [21] drew attention to the fact that an error has been generally committed in all previous quantitative comparisons of anisotropic signals, such as e.g., low-spin heme with a copper standard. We had used copper as well as cytochrome *c* standards for estimating the low-spin heme of cytochrome *c* oxidase. The values with the copper standard had been uniformly low and those with the heme standard relatively high, both centering around 35–40 % of the heme. After a re-evaluation of our data and new determinations based on the correct procedure, the deviations from the 50 % value are no longer such that the simplest assumption, namely that the low-spin heme represents 50 % of the heme of cytochrome *c* oxidase, can not be accepted.

RESULTS

Numerous records of titrations under different conditions of cytochrome *c* oxidase are in the literature. We have attempted in the experiments of Figs 1–3 and 5 and Table I to give a complete quantitative evaluation of the principal EPR signals of the enzyme at different oxidation states: with NADH and phenazine methosulfate as reductant (Figs 1 and 2, Table I), without (Fig. 1) and with (Fig. 2) cytochrome *c* present; with cytochrome *c* as reductant in the presence and absence of CO (Figs 3 and 4), with NADH and phenazine methosulfate as reductant and cytochrome *c* (Table I) or ferricyanide (Fig. 5 and Table II) as oxidant.

Reduction by NADH

The results of Fig. 1 generally confirm those obtained earlier [1] with EPR spectroscopy at 80 °K, except that, in the present work at 13 °K, higher precision, accuracy and resolution could be achieved. Thus the axial and rhombic components

* The value of 12.0 was given in ref. 12 for 605 nm. The author stated, however, that the maximum in the reduced state was at 604–605 nm. In our preparations the maximum was at 604 nm.

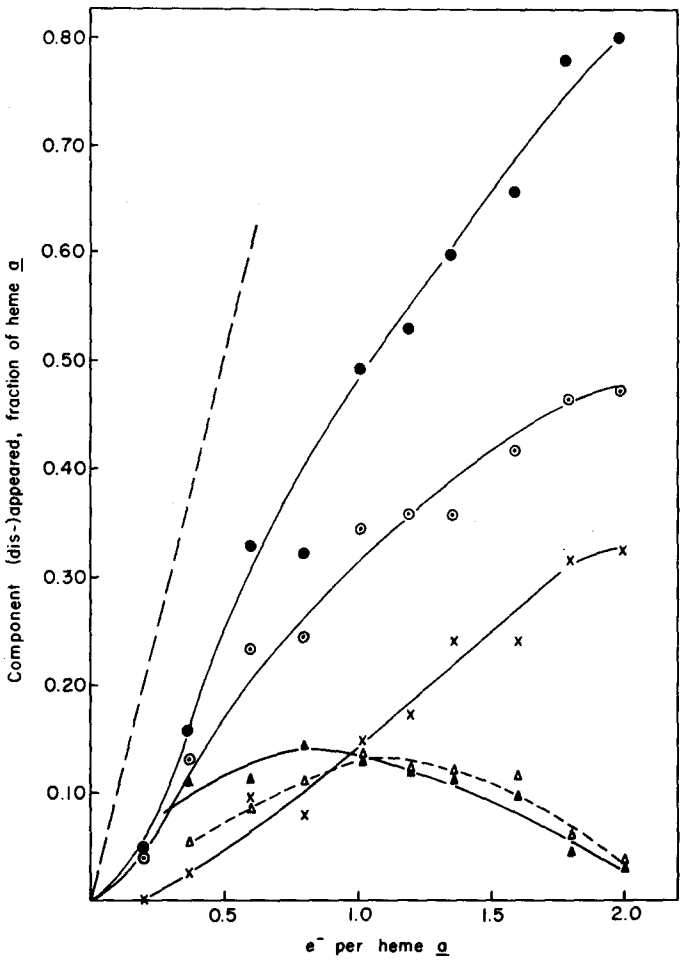

Fig. 1. Anaerobic titration of 0.64 mM cytochrome *c* oxidase with NADH in the presence of phenazine methosulfate. The abscissa shows reducing equivalents added per total heme *a* and the ordinate components which disappear or appear. Their concentration is expressed as fraction of total heme *a*. The broken line indicates the maximal amount of change that could occur in all components together, with the amount of reductant added. The symbols indicate: ⊙, low-spin heme signal disappeared; ×, copper signal disappeared; ●, sum of low-spin heme and copper signals disappeared; △, axial high-spin signal appeared; ▲, rhombic high-spin signal appeared.

of the ferric high-spin signal could be resolved and their quantitative evaluation improved. It is apparent that under the conditions of these titrations the axial and rhombic components represent approximately equal quantities of material, with a maximal amount of heme represented, in both signals together, of approximately 0.25 mol/mol of heme *a* present. Not considering the very initial period, when the high-spin signal has an altogether different shape (cf. ref. 6, Fig. 10, top curve) the rhombic signal reaches maximal development and decays somewhat earlier in the titration than the axial signal. The broken line in the graph shows total reducing equivalents added per heme *a* to indicate the maximal level to which all curves

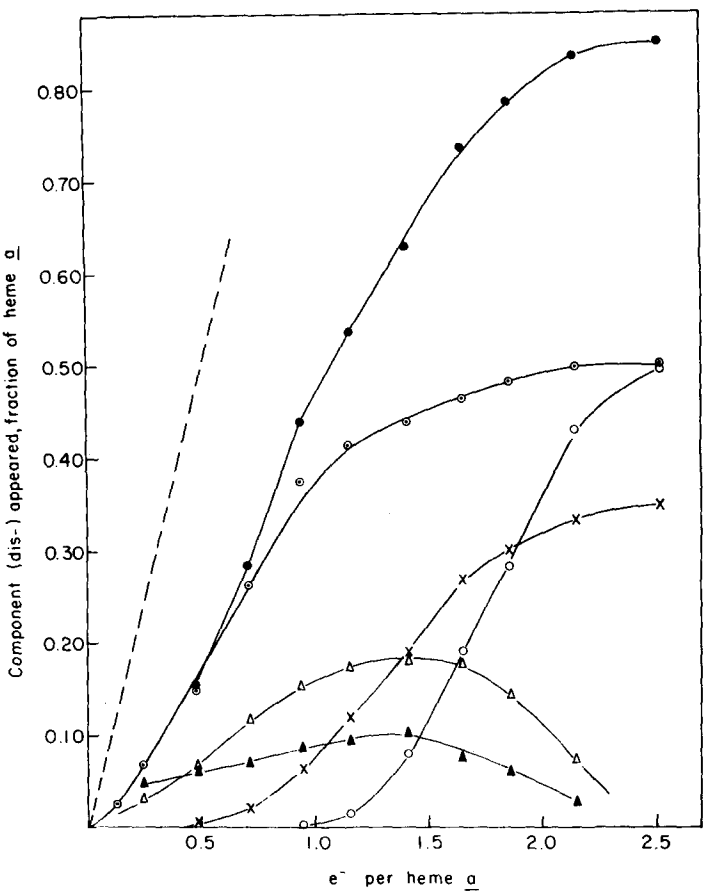

Fig. 2. Anaerobic titration of 0.50 mM cytochrome *c* oxidase with NADH in the presence of phenazine methosulfate and 0.25 mM cytochrome *c*. The presentation and symbols are analogous to those in Fig. 1. ○, cytochrome *c* reduced.

describing reduction of components could add up if all components were fully detectable by EPR. Fig. 2 is a repetition of the experiment of Fig. 1 with the same preparation used for Fig. 3, now in the presence of 0.50 mol of ferri-cytochrome *c* per mole of heme *a*. There is no significant change in the pattern of disappearance of the signal at $g = 3$ or the appearance of the high-spin signals. There is, however, a shift toward higher titrant concentrations in the disappearance of the copper and the high-spin signal*. The behavior of the high-spin signals, whose disappearance seems to reflect the reduction of a heme of low midpoint potential, is in line with

* In the experiment of Fig. 2, the ratio of the axial and rhombic high-spin signals is different from that in Fig. 1. This feature, however, is a property of the preparations used and is unrelated to the presence of cytochrome *c*. The quantitative relationship of the high-spin signals in Fig. 2 is the same as in Fig. 3, where the same preparation has been used. The experiment of Fig. 2 was also carried out with the preparation used for Fig. 1 and in this instance the high-spin signals showed the quantitative relationship of Fig. 1.

observations made in potentiometric [4, 13] and coulometric [14] titrations followed optically.

Reduction by cytochrome c

Fig. 3 shows a titration with cytochrome *c* as reductant at pH 7.4 and points obtained at two cytochrome *c* levels at pH 9.3 and with CO present at pH 7.4. It is apparent that 1 mol of reduced cytochrome *c* per mol of heme *a* abolishes 80 % of the low-spin heme signal and decreases the copper signal by ~ 15 %. It is interesting to compare the quantity of cytochrome *c* oxidized during this titration with the sum

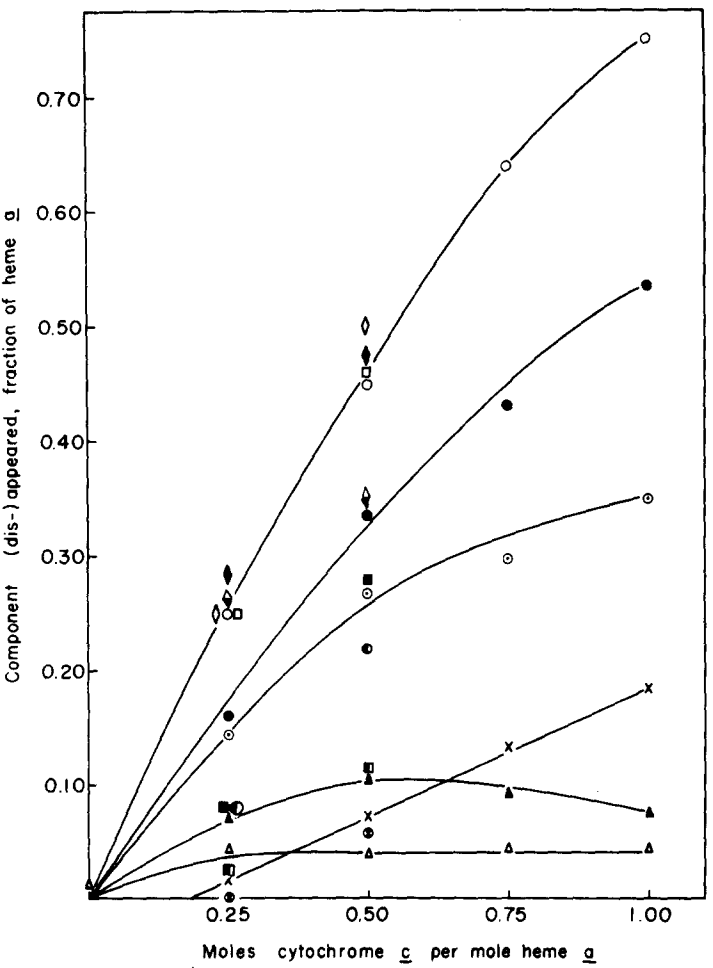

Fig. 3. Anaerobic titration of 0.2 mM cytochrome *c* oxidase with 2.5 mM reduced cytochrome *c*. The presentation and symbols are analogous to those of Figs 1 and 2 except that ○ indicates cytochrome *c* oxidized. The abscissa shows mol cytochrome *c* added/mol total heme *a*. Additional symbols are: □, cytochrome *c* oxidized; ◐, low-spin signal disappeared; ⊗, copper signal disappeared; ■, sum of low-spin and copper signals disappeared, all in the presence of CO; ◇, ◈, ◧, ◆, cytochrome *c* oxidized, low-spin, copper and sum of both signals disappeared, respectively, in the absence of CO but at pH 9.3 in Tris buffer.

of low-spin heme and copper signals that disappear. There is a widening gap between these two quantities indicating that, if signal disappearance does indeed mean that these components are reduced, additional, EPR-undetectable electron acceptors in the enzyme must be reduced simultaneously.

Reflectance spectra obtained from this experiment are shown in Fig. 4. The top curve (A) shows the spectrum of the oxidized sample. After addition of 0.5 mol of *c* per mol of heme *a*, all *c* is oxidized and the 655 nm absorption is barely decreased (B), while with 1 mol of *c* per mol of heme *a* little cytochrome *c* remains reduced but the 655 nm absorption is substantially diminished (C). With 1 mol of *c* in the presence of CO (D) this band is decreased to a larger extent than with 1 mol of *c* per heme *a* in the absence of CO (C), while according to EPR less of the low-spin signal disappears in the presence of CO. Cytochrome *c* is oxidized, as with 1 mol of *c* per heme *a* in the absence of CO. This suggests that, in the presence of CO, electrons are diverted towards the component(s) represented in the 655 nm absorption, with the low-spin heme remaining more oxidized.

The experiments at pH 9.3 are particularly significant from the standpoint of the total balance of oxidation-reduction since the low-spin heme signal is more

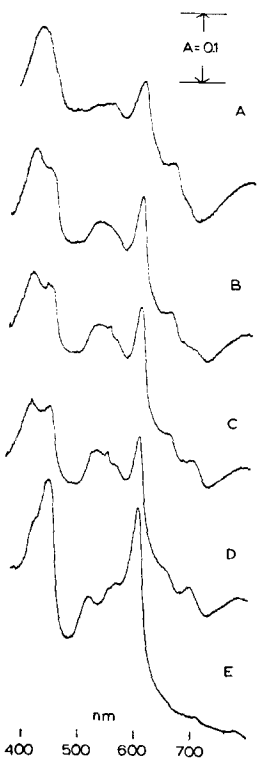

Fig. 4. Low-temperature (100 °K) reflectance spectra from samples of the experiment of Fig. 3. (A), oxidized sample in the absence of cytochrome *c*; (B), after addition of 0.5 mol of reduced cytochrome *c* per mol of heme *a*; (C), after addition of 1 mol of reduced cytochrome *c* per mol of heme *a*; (D), as (C) but in the presence of CO; (E), completely reduced with an excess of NADH plus phenazine methosulfate.

TABLE I

BALANCE OF OXIDATION-REDUCTION ON ANAEROBIC EQUILIBRATION OF CYTOCHROME *c* OXIDASE, CYTOCHROME *c* AND/OR NADH IN THE PRESENCE AND ABSENCE OF CO

These experiments were conducted in tubes described in ref. 10. The enzyme was placed in the main sidearm, NADH and phenazine methosulfate in the minor sidearms and cytochrome *c* at the bottom of the EPR tube. The reductive phase was carried out as described [1] in 0.1 M phosphate at pH 7.2 and 18 °C for 15 min, either in vacuo or in the presence of CO. The contents of all compartments were then thoroughly mixed and light absorption spectra were recorded at room temperature. The samples were then frozen for EPR and reflectance spectroscopy. No significant differences were observed in the oxidation state of the components by spectrophotometry at 18 and −180 °C. The calculation of the results was based on a millimolar absorption of NADH at 340 nm of 6.22 $mM^{-1} \cdot cm^{-1}$ and the assumption that all NADH so determined is active as reductant, donating 2 electron equivalents per mol. Cytochrome *c* oxidase was assumed to contain 2 oxidizing equivalents per heme *a* and cytochrome *c* 1 equivalent.

	Total oxidizing or reducing equivalents in components mixed			presence of CO	Distribution of oxidizing or reducing equivalents after equilibration							
	Cytochrome *c* oxidase oxidized* (nequiv.)	Cytochrome *c* oxidized (nequiv.)	NADH reduced† (nequiv.)		Cytochrome *c* reduced	Low-spin heme‡ disappeared		Copper disappeared‡		High-spin signal appeared**		
						(%)	(nequiv.)	(%)	(nequiv.)	axial (nequiv.)	rhombic (nequiv.)	total (nequiv.)
1	197	0	150	−	0	100	49.3	100	34.5	0.7	0	0.7
2	197	0	125	−	0	94	46.2	82.5	28.4	3.2	4.1	7.3
3	197	0	100	−	0	91.5	43.5	61	21.0	5.3	7.9	13.2
4	197	0	75	−	0	83.0	41.0	37.5	12.9	4.6	8.6	13.2
5	197	128	150	−	21.5	88.5	43.9	42	14.5	4.9	7.7	12.6
6	197	128	150	+	28.0	73.5	36.1	29	10.0	0.4	2.0	2.4††
7	197	210	150	−	27.0	86.5	42.5	39	13.5	4.7	7.9	12.6
8	197	128	197	−	36.5	90.0	44.3	55	19.0	5.9	7.5	13.4
9	197	128	197	+	41.0	83.5	41.1	46.5	16.0	0.3	0.6	0.9††

* This includes 2 hemes and 2 copper atoms. The total volume was 0.5 ml.

** Determined by integration, cf. ref. 1.

† 3 μM phenazine methosulfate was added with NADH.

†† These values are probably fortuitous, depending on the light falling on the sample during freezing.

‡ The number of nmol was calculated on the assumption (see text) that 0.35 mol of copper and 0.50 mol of low-spin heme per heme *a* is represented by the signals observed in the oxidized blank sample.

extensively diminished. Data collected for samples at pH 6.06 in 0.04 M phosphate buffer (not shown in Fig. 3 to avoid congestion) showed that at 0.23 and 0.46 mol of reduced cytochrome *c* per mole of heme *a*, respectively, signal intensity equivalent to 0.1 and 0.2 mol low-spin heme and 0.0 and 0.01 mol of copper disappeared per heme *a*, while high-spin signals equivalent to 0.1 and 0.2 mol/mol of heme *a* appeared with 0.23 and 0.46 mol of cytochrome *c* added, respectively. The last two samples showed one third of the high-spin signal intensity in the rhombic and two thirds in the axial components. The added cytochrome *c* was completely oxidized in either case. It thus appears that at the more acid pH the electrons that have entered cytochrome *c* oxidase are to an even larger extent located in the undetectable components of high potential than at neutral pH.

It is also interesting to note that at the lower pH the signal intensity of the high-spin signal is predominantly in the axial component in contrast to the situation at pH 7.4. This axial component has a broader signal than that seen at pH 7.4 and resembles that shown in Fig. 11B rather than that of Fig. 14A of ref. 6. The shape, type and intensity of the high-spin signals obviously depends on pH.

We carried out pH-jump experiments in order to test whether there is rapid equilibration between the high-spin signal types. Cytochrome *c* oxidase was reduced anaerobically at pH 7.4 by 1 mol of reduced cytochrome *c* per mol of heme *a* and loaded into a syringe of the rapid reaction apparatus. Large axial and rhombic high-spin signals were observed as usual. This solution was then rapidly mixed with 0.1 M acetate buffer of pH 4.2 or 0.1 M citrate buffer of pH 3, so that a final pH of approx. 6 was reached. Within 6 ms the rhombic and axial signals had reached their final decreased and increased levels, respectively. The axial signals, however, did not change their shape.

Reoxidation with cytochrome c or ferricyanide

Table I is a summary of experiments conducted under a sufficient number of different conditions that presentation in a figure is not very useful. For these experiments cytochrome *c* was used as oxidant after reduction of the enzyme by 1.5 and 2 electron equivalents (NADH and phenazine methosulfate) per mol of heme *a*, in the presence and absence of CO. It is again apparent that the low-spin heme and copper signals are diminished to a lesser extent and more cytochrome *c* is reduced in the presence of CO. The experiment of Fig. 5 is similar to that of Table I, except that ferricyanide was used as oxidant rather than cytochrome *c* and CO was not present. Oxidized cytochrome *c* had been added in one set of samples, which then received an additional amount of reductant so that in each sample initially both cytochrome *c* and cytochrome *c* oxidase were exactly fully reduced. The samples were then titrated back with ferricyanide. As in the reductive phase, an extra amount of titrant was added to the samples containing *c*, equivalent to the amount of cytochrome *c* present. Fig. 5 shows that the copper signal was restored with 1 oxidizing equivalent per heme. The low-spin heme signal reappeared completely only after addition of 3 to 4 equivalents. Cytochrome *c* (not shown) was $> 90\,\%$ reoxidized after 1.65 equivalents had been added. There was no significant difference in the behavior of the low-spin heme or copper, whether cytochrome *c* was present or not. As always with ferricyanide oxidation, large rhombic high-spin signals were observed and the most significant difference brought about by the presence of cytochrome *c*

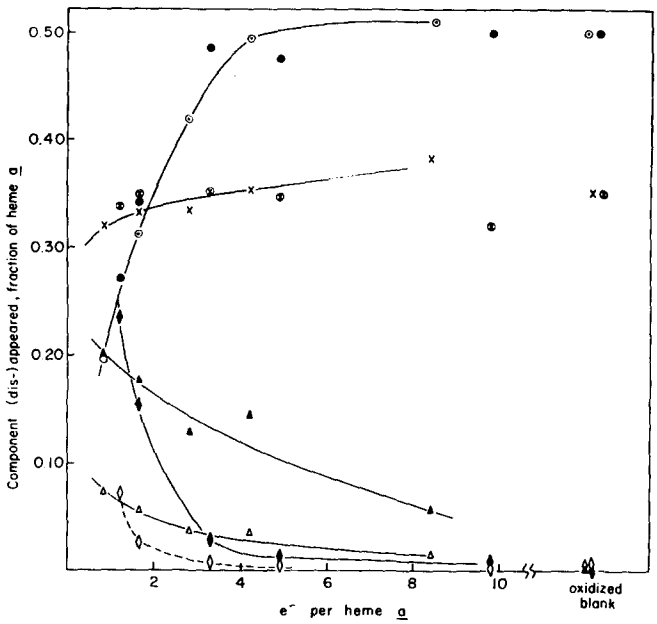

Fig. 5. Anaerobic oxidative titration with 15 mM ferricyanide of 96 μM cytochrome *c* oxidase previously reduced (2 equivalents per heme *a*) by NADH, and phenazine methosulfate. In one set of samples 35 μM cytochrome *c* was present. The concentrations of NADH and ferricyanide were adjusted so as to account for the additional reducing or oxidizing equivalents needed for cytochrome *c*. The presentation is analogous to that of Figs 1–3. The abscissa shows oxidizing equivalents added per heme *a*. The symbols in the absence of cytochrome *c* are: ⊙, low-spin signal restored; ×, copper signal restored; △, axial high-spin signal; ▲, rhombic high-spin signal. In the presence of cytochrome *c* the corresponding symbols are (in the same order): ●, ⊗, ◇, ◆.

seems to be that both axial and rhombic high-spin components disappear at lower ferricyanide concentrations. There is no obvious explanation or reported precedent to this observation of which we are aware. Reflectance spectra show (cf. ref. 5, Fig. 8c) that, after reoxidation by ferricyanide under the conditions of Fig. 5, the absorption band at 655 nm is very weak. The accumulation of rhombic high-spin ferric heme and the essential absence of the 655 nm absorption appear to indicate that under the conditions used ferricyanide is unable to restore the original oxidized state of cytochrome *c* oxidase (cf. Schroedl, N. and Hartzell, C. R., manuscript in preparation).

In experiments analogous to those of Fig. 5, the enzyme was reduced (NADH and phenazine methosulfate), and then reoxidized by ferricyanide in the presence and absence of CO. Whereas in the absence of CO apparent reoxidation proceeds as indicated in Fig. 5 and discussed above, in the presence of CO the so-called 'mixed valence' state of Greenwood et al. [15] results, the spectrum of which is shown in Fig. 6C. This spectrum is decidedly different from that of the oxidized or reduced form (Fig. 6A, B). Significant is the absence of the 655 nm absorption, indicating that, although the low-spin heme and copper signals are largely restored, one or both of the other electron acceptors of the enzyme are still in the reduced state.

TABLE II

ANAEROBIC REOXIDATION OF LOW-SPIN HEME AND COPPER WITH FERRICYANIDE AFTER REDUCTION WITH NADH IN THE PRESENCE OF CO

These experiments were conducted in tubes described in ref. 20, which in addition had two minor sidearms offset by 90° with respect to the main 'crusher'-type sidearm. The tube was evacuated repeatedly and filled with CO. The reductive phase was carried out as described for Table I. Care was taken to keep a small evacuated bulb containing the dried ferricyanide solution in the main sidearm. After reduction the bulb was crushed and the contents of the tube were thoroughly mixed in the part containing the various compartments and then made to run down into the EPR observation tube. For the evaluation the assumptions mentioned in the legend to Table I were made. The quantity of ferricyanide was determined roughly by pipetting of a solution into the bulb and exactly by weighing the bulb before and after addition of the solution.

	Total oxidizing or reducing equivalents in components mixed		Signal intensity remaining after reduction**				Ferricyanide added†† (nequiv.)
	Cytochrome *c* oxidase oxidized* (nequiv.)	NADH† (nequiv.)	Cu		Low-spin heme		
			(%)	(nequiv.)	(%)	(nequiv.)	
1	200	158	1	0.35	1	0.5	25
2	500	324	24	21	10	12.4	62
3	500	346	12	10.5	5	6.1	65
4	500	370	6	5.2	3	3.7	63.5
5	500	370	3	2.6	1.5	1.85	66

Signal intensity restored by ferricyanide				Cu + low-spin heme (nequiv.)	Cu represented in restored signal, calculated for Cu^{2+}-Cu^{2+}-pair*** (nequiv.)	Cu represented in restored signal, calculated for Cu^{2+}-Cu^{2+}-pair + low-spin heme (nequiv.)	Excess of total nequiv. restored (last column) over ferricyanide added (%)
Cu		Low-spin heme					
(%)	(nequiv.)	(%)	(nequiv.)				
37	13	7	3.6	16.6	37	40.5	62
31.5	27.5	13.5	17	44.5	79	96	55
42	37	18.5	23	60	105	128	97
39	34	15	18.5	52.5	97.5	116	83
33	29	10.5	13	42	82.5	95.5	45

* This includes 2 hemes and 2 copper atoms. The total volume was 0.5 ml. Two different preparations were used in which the inactive copper was less than 5 % of the total copper.

** The number of nanoequivalents was calculated on the assumption (see text) that 0.35 mol of copper and 0.50 mol of low-spin heme per mol of heme *a* is represented by the signals observed in the oxidized control sample.

*** Calculated on the assumption that the copper signal observed in the oxidized control represents 100 % of both copper atoms in the enzyme and that the observed low intensity in the control is due to interaction of these copper atoms.

† Phenazine methosulfate was added with NADH to a final concentration of 3 μM.

†† Ferricyanide was added in the closed CO filled tube [20] by crushing a small evacuated glass bulb containing an evaporated aliquot of a ferricyanide solution.

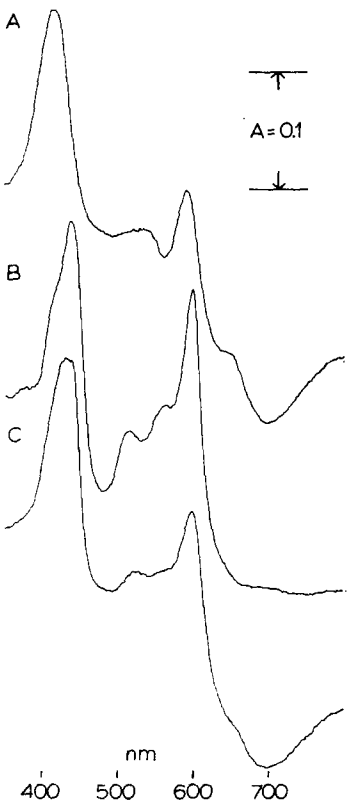

Fig. 6. Low-temperature reflectance spectra of cytochrome *c* oxidase, reoxidized by ferricyanide in the presence of CO after previous reduction by NADH and phenazine methosulfate. Enzyme, 77 μM, was reduced anaerobically with 2 equivalents per heme *a* in the presence of CO and then reoxidized with 2.6 equivalents of ferricyanide per heme *a*. The spectra represent, from top to bottom: oxidized blank; reduced in the presence of CO; reoxidized with ferricyanide to the 'mixed valence' state [15].

Since the copper signal of cytochrome *c* oxidase has unusual characteristics as compared to signals observed with other copper proteins or copper complexes of low molecular weight, the possibility has been considered that this signal may represent a pair of interacting copper atoms. Experiments of the type just described have a bearing on this point. When cytochrome *c* oxidase, after reduction with NADH and phenazine methosulfate in the presence of CO, is oxidized with small increments of ferricyanide, the copper signal is very readily restored, as shown in Table II. The resulting balance of oxidation-reduction of the components of the system seems incompatible with the notion that 100 % of the copper present in the enzyme is represented in the copper signal as a pair of interacting cupric ions. More signal is restored than the quantity of ferricyanide added could account for according to this model.

It has been shown previously [1] that no changes in cytochrome *c* oxidase can be detected by EPR after addition of azide, unless the enzyme is reduced. At

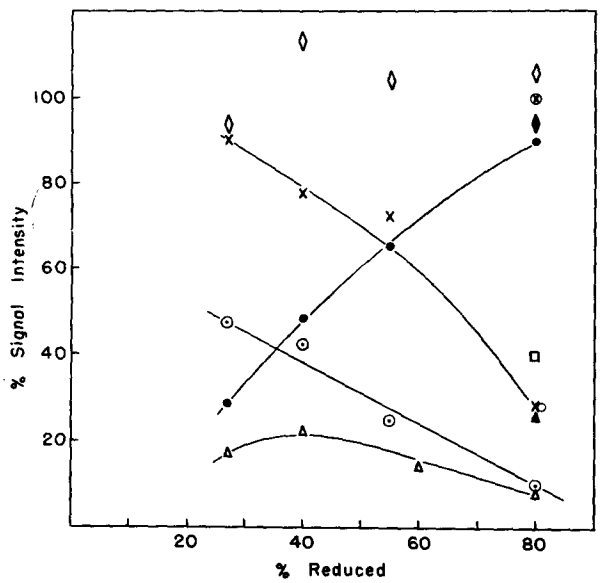

Fig. 7. Reduction and reoxidation of cytochrome *c* oxidase in the presence of azide. Enzyme, 73 μM in the presence of 29 mM sodium azide, was reduced by NADH and phenazine methosulfate as indicated on the abscissa; assuming 100 % reduction at 2 equivalents per heme *a*. One sample, after reduction by 80 %, was reoxidized by 0.8 equivalents of ferricyanide. (At higher levels of ferricyanide no significant signals at $g = 2.9$ or 2.75 remained and the signal at $g = 3$ was restored to its original intensity before reduction; this is not shown in the figure.) The ordinate shows % of maximal signal intensity normalized to the intensity of the signal at $g = 3$ in the original sample. The symbols are: ○, low-spin signal at $g = 3.0$; ●, low-spin signal at $g = 2.9$; △, low-spin signal at $g = 2.75$; ◇, sum of intensities of signals at $g = 3.0$, 2.9 and 2.75; ×, copper signal; □, ○, ▲, ◆, ⊗, the corresponding signals after 80 % reduction and subsequent addition of 0.8 equivalent of ferricyanide per mol of heme *a*.

least three different signals appear at $g \sim 3$ at states of partial reduction [1, 16], namely the usual low-spin signal at $g = 3$ and a major ($g = 2.9$) and minor ($g = 2.75$) signal typical of the presence of azide. Cytochrome *c* oxidase was reduced in the presence of 29 mM sodium azide to several states by NADH and phenazine methosulfate. Fig. 7 shows the intensities in these reduced samples of the usual low-spin signal at $g = 3$ and the signals produced by azide at $g = 2.9$ and 2.75, relative to the signal at $g = 3$ in the original oxidized sample, which is taken as 100 %. When at the state of 40 and 80 % reduction 2.7 equivalents of ferricyanide per mol of heme *a* were added from a sidearm, the signals at $g = 2.9$ and 2.75 were abolished and the signal at $g = 3$ reappeared in its original intensity (not shown in Fig. 7). When at 80 % reduction, 0.8 equivalents of ferricyanide per mol of heme *a* was added, this reversal was only partial, as shown in Fig. 7 by the symbols □, ○, ▲, ◆ and ⊗. The symbol ◇ shows the sum of the intensities represented in the 3 signals at $g = 3$, $g = 2.9$ and $g = 2.75$ after partial reduction and after partial reoxidation of the sample reduced to 80%. It is apparent from the figure that, over the range of 27–80% reduction covered, the sum of the intensities of these signals remains constant within error. This is the range of reduction over which, in the absence of azide, the intensity

of the high-spin signal at $g = 6$ is usually found to be fairly constant in titration (cf. Figs 1 and 2).

DISCUSSION

A comprehensive discussion of the results of the three papers of this serie [6, 7] will be found at the end of the accompanying paper [7]. The present discussion will therefore be limited to points related specifically to some of the experiments described here.

In a comparison of the experiments of Figs 1 and 2 on the titration of cytochrome *c* oxidase with NADH in the presence and absence of cytochrome *c*, it may seem unexpected at first sight that the copper signal disappears only at substantially higher concentrations of titrant when cytochrome *c* is present. According to potentiometric titrations the midpoint potential of this component is little, if at all, affected by addition of cytochrome *c* [4]. However, if the midpoint potentials of the high-potential heme and possibly also the high-potential copper component are raised, this may manifest itself in a shift of the reduction of the detectable copper toward higher titrant concentrations. It is also possible that part of the detectable (low-potential) copper which becomes apparent only under special conditions (e.g. re-oxidation with ferricyanide, see below) may be reduced initially or may replenish the pool of the detectable copper.

We have performed experiments in collaboration with Dr J. Anderson and T. Kuwana, in which samples were reduced by electro-generated benzyl viologen radical [17] and examined optically at room temperature, and thereafter by low-temperature EPR and optical reflectance spectroscopy. The EPR and reflectance spectra were analogous to those observed by us for the corresponding oxidation states in the experiment of Fig. 1. We feel justified to conclude, therefore, that the midpoint potentials calculated from the electrochemical data of Kuwana and collaborators [14, 18] are applicable in our titrations as well. We have independently attempted to simulate the titration curves shown in Figs. 1–3. The best fits to the experimental curves were obtained with the following values for the respective midpoint potentials: Fig. 1 (based on an assumed $E_{m\,7.4}$ for the disappearance of the low-spin signal of +340 mV), EPR detectable copper, +260 mV; high spin heme appearance, +345 mV, disappearance, +210 mV. Fig. 2 (based on an assumed $E_{m\,7.4}$ for cytochrome *c* of +230 mV), low-spin heme, +330 mV; EPR detectable copper, +260 mV; high spin heme appearance, +345 mV; disappearance, +200 mV. Fig. 3 (basis as Fig. 2), low-spin heme, +340 mV; EPR detectable copper, +260 mV; high spin heme appearance, +325 mV. We estimate that the uncertainty of these values is ±20 mV. They are in satisfactory agreement with those derived by spectrophotometry at room temperature. However, it is noteworthy that in the experiments of Fig. 2 and 3 and in Table I the EPR detectable copper consistently has a midpoint potential higher than that of cytochrome *c*. For this reason, we have used a value of +230 mV of cytochrome *c* in our simulations. This is the main discrepancy from the generally accepted values which we have observed and we consider the possibility that at the low temperature, at which EPR measurements are made, the midpoint potentials may not be the same as derived from spectrophotometry at room temperature. The discrepancy in the reduction of the copper observed in rapid reaction

studies by low temperature and room temperature spectrophotometric studies, as reported in the accompanying paper [7], may at least in part have a similar explanation.

Evidence was presented above, particularly in the experiment of Figs 3 and 4, on reduction of the enzyme by reduced cytochrome *c*, that, in addition to the components observable by EPR, EPR-undetectable electron acceptors must be reduced to a variable degree, depending on conditions. As will be elaborated in the accompanying paper [7], there is a relationship between this apparent additional reduction, the disappearance of the absorption band at 655 nm (cf. Fig. 4), and the appearance of the high-spin signal.

When the same experiment was conducted at pH 9.3 electrons from cytochrome *c* go more extensively to the low-spin heme and copper rather than to the two other less readily detectable components. This would be in line with the reported change of midpoint potential of the high-potential heme component with pH [13]. However, changes in the 655 nm absorption on reduction at pH 9.3 indicate that some changes in the electron acceptor(s) represented by this absorption band also occur. The absorption at 655 nm is less pronounced at high pH and is therefore difficult to evaluate.

The observation made in the experiment of Fig. 7 (reduction and reoxidation in the presence of azide), namely that the three signals at $g = 3$, $g = 2.9$ and $g = 2.75$ together show an approximately constant intensity over a broad range of oxidation states, may of course be fortuitous. If it is not, it suggests a relationship between the components of the enzyme that are represented in these signals. The simplest assumption, analogous to the proposal that the signals at $g = 3$ and $g = 6$ originate from different states of the same component [5], is that all three signals originate from the same component, which would mean that apparently this component is not reduced at all, unless rapid reduction and reoxidation occur in the time span before our observations are made. According to this view the action of azide would become manifest in an accumulation of one of the hemes in an oxidized state, from which it is not readily reduced. This agrees with our findings in previous titrations [1].

Other conclusions and inferences which we would like to draw from the experiments reported in this paper will be presented and discussed in the context of additional observations which were made in the course of rapid kinetic studies [7].

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REFERENCES

1 van Gelder, B. F. and Beinert, H. (1969) Biochim. Biophys. Acta 189, 1–24
2 Malmström, B. G. (1974) Q. Rev. Biophys. 6, 389–431
3 Wilson, D. F. and Leigh, Jr, J. S. (1972) Arch. Biochem. Biophys. 150, 154–163
4 Tiesjema, R. H., Muijsers, A. O. and van Gelder, B. F. (1973) Biochim. Biophys. Acta 305, 19–2
5 Hartzell, C. R., Hansen, R. E. and Beinert, H. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2477–248
6 Hartzell, C. R. and Beinert, H. (1974) Biochim. Biophys. Acta 368, 318–338
7 Beinert, H., Hansen, R. E. and Hartzell, C. R. (1976) Biochim. Biophys. Acta 423, 339–35
8 Orme-Johnson, N. R., Hansen, R. E. and Beinert, H. (1974) J. Biol. Chem. 249, 1928–1939
9 Orme-Johnson, N. R., Hansen, R. E. and Beinert, H. (1974) J. Biol. Chem. 249, 1922–1927
10 Hansen, R. E., van Gelder, B. F. and Beinert, H. (1970) Anal. Biochem. 35, 287–292
11 Palmer, G. and Beinert, H. (1964) Anal. Biochem. 8, 95–103
12 van Gelder, B. F. (1966) Biochim. Biophys. Acta 118, 36–46
13 Wilson, D. F., Lindsay, J. G. and Brocklehurst, E. S. (1972) Biochim. Biophys. Acta 256, 277–28
14 Mackey, L. N., Kuwana, T. and Hartzell, C. R. (1973) FEBS Lett. 36, 326–329
15 Greenwood, C., Wilson, M. T. and Brunori, M. (1974) Biochem. J. 137, 205–215
16 Wever, R. and van Gelder, B. F. (1974) Biochim. Biophys. Acta 368, 311–317
17 Hawkridge, F. M. and Kuwana, T. (1973) Anal. Chem. 45, 1021–1027
18 Heineman, W. R., Kuwana, T. and Hartzell, C. R. (1972) Biochem. Biophys. Res. Commun. 49 1–8
19 Aasa, R., Albracht, S. P. J., Falk, K.-E., Lanne, B. and Vänngård, T. (1976) Biochim. Biophys Acta 422, 260–272
20 Orme-Johnson, W. H. and Beinert, H. (1969) Anal. Biochem. 32, 425–435
21 Aasa, R. and Vänngård, T. (1975) J. Magn. Res., in press