Magnetic Properties of Iron and Copper in
Cytochrome Oxidase

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The paramagnetic susceptibilities of the transition metal components (cytochromes a and a_3 and copper) of cytochrome oxidase, oxidized, reduced and in the presence of cyanide and carbon monoxide, have been determined. Assuming that reduced cytochrome a_3 is the only component responsible for the volume magnetic susceptibility changes of the reduced enzyme preparation upon the addition of cyanide or carbon monoxide, the results obtained with a densitive volume magnetic susceptometer and electron spin resonance spectrometers are analyzed. Cytochrome a_3 in the oxidized form has a molar susceptibility of 7 900 $\times$ 10^{-6} cgs emu, which is intermediate between known data of oxidized hemoproteins of the low and high spin types, whereas cytochrome a is a hemoprotein of the low spin type magnetically similar to cytochrome c. Although copper is found to be responsible for part of the susceptibility changes in some part of the results, no evidence in favour of a role of copper in the oxidase mechanism is found.

$\mathbf{P}$urified preparations of cytochrome oxidase contain two different cytochromes designated a and a_3[1,2]. Cytochrome a is inert to cyanide, carbon monoxide and oxygen, whereas cytochrome a_3 reacts with these compounds. In addition, since the carbon monoxide compound of reduced cytochrome a_3 is photodissociable[3] and its absorption spectrum is identical with the photochemical action spectra of the carbon monoxide-inhibited cellular inhibition[4], cytochrome a_3 is considered to be *Atmungsferment* of mammalian tissues[1-3]. Purified preparations of cytochrome oxidase also contain copper in an atomic ratio to the iron of haemin a which according to different investigators varies from 1 to 2[5-8]. Although it was demonstrated that the copper in the oxidized preparation is in the cupric state and can be partially reduced by various reducing systems[6,7], it has been suggested that the rapid electron donation to oxygen is accountable in terms of the heme and not the copper portion of the oxidase preparation[9,10]. It has further been proved that most of the copper is not required for the enzyme activity of cytochrome oxidase[11]. The magnetic susceptibility change during the reaction of cytochrome oxidase was

previously investigated [10] by using a sensitive magnetic susceptometer combined with a regenerative rapid flow system [12] in which an increase in volume magnetic susceptibility was observed in the reaction of reduced cytochrome oxidase with oxygen.

The present investigation of the magnetic properties of a cytochrome oxidase preparation was undertaken to measure more accurately the magnetic susceptibility of the preparation with the aim to elucidate the properties and mode of action of the iron of cytochromes a and a_3 and of the copper in connection with the previous magnetic study [10]. Because of the complexity of the system it was important that determination of the bulk magnetic susceptibility in solution could be combined with a study of the electron spin resonance (ESR) absorption due to the copper to monitor changes of the copper valency as described by Malmström *et al.*[13]

EXPERIMENTAL

Material. Purified cytochrome oxidase was prepared from beef heart as described by Yonetani [2] and was dialysed for 72 h against 0.05 M phosphate buffer, pH 7.2, containing 1 % Emasol 4130 [14]. Aliquots of this buffer were taken and used as reference solutions throughout the work. In every case the reference solution was given the same additions and treated in the same way as the sample. The dry weight of cytochrome oxidase in the stock solution was determined to be 94.0 mg/ml. The partial specific volume was measured to be 0.81 ($\pm$ 0.02) ml/g by weighing several samples of the stock solution, of the buffer and of water, taken by a micropipet. The iron content was found to be 510 μM by a modification of Lorber's method with sulfosalicylic acid [15]. Based on this analysis the extinction coefficient at 605 mμ was found to be 21.0 (mM Fe)$^{-1}$ cm^{-1} [8]. Copper was determined from the colour (measured at 450 mμ) developed by addition of diethyldithiocarbamate to the sample, after previous digestion with H_2SO_4 and H_2O_2, and additions of pyrophosphate and ethyl alcohol according to a slight modification of the standard procedure [16]. The stock solution contained 1025 μM Cu which means that the atomic ratio Cu/Fe of the sample was 2:1. No part of this copper could be removed by dialysis against 0.5 mM KCN for 48 h. The ratio of the increase in optical density at 445 and 605 mμ upon reduction with dithionite was 6.1. The cytochrome c used was made by Sigma Type III, from horse heart.

Methods. The volume susceptibility of the samples was investigated at 20°C by means of the sensitive susceptometer balance constructed by Theorell and Ehrenberg [17] and equipped with an improved temperature control. The instrument was calibrated with a carefully analyzed solution of $NiCl_2$ assuming $\chi_{NiCl_2} = 4434 \times 10^{-6}$ cgs emu.

The measurements of ESR absorption were made at the temperature of liquid nitrogen and at room temperature. The low temperature measurements were made with an X-band spectrometer with 70 cycles field modulation, constructed by one of the authors (A.E.), and with the Varian X-band spectrometer V-4500 equipped with a 100 kc field modulation unit. The room temperature experiments could only be made at the higher sensitivity obtained with the latter instrument. Quartz sample tubes with 3 mm inner diameter were used at liquid nitrogen temperature and with 1 mm inner diameter at room temperature. Diphenylpicrylhydrazyl with $g = 2.0036$ was used as a g-value reference.

All samples were made anaerobic by rapid evacuation in Thunberg tubes and twice flushing with nitrogen or argon. Reduction was achieved by the addition of 10 μl 15 % $Na_2S_2O_4$ per ml sample, reaction with cyanide by the addition of 10 μl 0.3 M KCN per ml sample, and reaction with CO by flushing the Thunberg tube with this gas. Any contact with air was avoided as far as possible during the filling of the sample tube of the susceptometer balance or of the ESR spectrometer. When necessary, complete anaerobiosis was achieved in the ESR experiments by attaching the sample tube *via* a tygon tubing to a separate outlet of the Thunberg tube, and washing the argon with pyrogallol.

Table 1. Experimental susceptibility data on the investigated cytochrome oxidase samples, and tentative molar susceptibilities of the transition metal components. The errors given are estimated maximal deviations. For comparison data on haem a treated as a single haemoprotein, cytochrome A, have been included. A correction of 290×10^{-6} cgs emu has to be added to the susceptibilities of a, a_3 and A according to Griffith.[24]

Sample No.	Additions made to oxidized cytochrome oxidase	Instrument reading Scale divisions	χ mean $\times 10^6$ cgs emu $a + a_3 + Cu$ (1535 μM)	Cu (1025 μM)	$\chi_M \times 10^6$ cgs emu as two different cytochromes haem a — a (330 μM)	a_3 (180 μM)	as a single haemoprotein Cytochrome A (510 μM)
1	None	$-4\ (\pm 2)$	2 470 (± 50)	1 500 (Cu'')	2 400(a''')	7 900(a_3''')	4 300 (A''')
2	KCN	$-48\ (\pm 2)$	1 840 (± 50)	1 500 (Cu''—CN)	2 400(a''')	2 400(a_3'''—CN)	2 400 (A'''—CN)
3	Na$_2$S$_2$O$_4$	$-79\ (\pm 10)$	1 400 (± 50)	0 (Cu')	0 (a'')	12 000(a_3'')	4 200 (A'')
4	KCN + Na$_2$S$_2$O$_4$	$-169\ (\pm 10)$	130 (± 150)	0 (Cu') or (Cu'—CN)	0(a'')	2 400(a_3'''—CN)	0 (A''—CN)
5	Na$_2$S$_2$O$_4$ + KCN	$-171\ (\pm 2)$	100 (± 50)	0 (Cu') or (Cu'—CN)	0(a'')	0(a_3''—CN)	0 (A''—CN)
6	Na$_2$S$_2$O$_4$ + CO	$-178\ (\pm 2)$	0	0 (Cu') or (Cu'—CO)	0(a'')	0(a_3''—CO)	0 (A''—CO)

RESULTS AND DISCUSSION

The readings on the susceptometer balance for the six different samples are given in column 3 of Table 1.

The interpretation of the experimental data on cytochrome oxidase is difficult (a) because multiple components are involved and (b) because the separate molar magnetic susceptibilities of the components have not yet been determined. In order, however, to make a preliminary interpretation of the data, it is desirable to make a series of assumptions and to determine what conclusions can be made concerning the nature of cytochrome oxidase on the basis of such assumptions. The first assumption with regard to the data of Table 1 is that Cu' * and its hypothetical CO compound in samples 3 and 6 (cf. column 5) are diamagnetic, since other cuprous compounds are known to be diamagnetic [18,19]. Secondly, it is assumed that haem-a-CO compounds are diamagnetic, since this is a property common to all hitherto investigated haem-CO compounds. The experimental observation that there is a great difference of magnetic susceptibility between samples 3 and 6 indicates that a high spin haem is involved. If one computes the molar magnetic susceptibility from the data on samples 3 and 6 on the basis of the total haem content of the preparation (510 mM) a value of $4\ 200 \times 10^{-6}$ cgs emu is obtained for A'' (cf. column 8). This value differs from that of any known ferro-haemoprotein (haemoglobin, [20], myoglobin [21], leghaemoglobin [22] and horse radish peroxidase [23]). It is, therefore, more appropriate to consider (a) that only a part of the total reduced haem-a is in the high spin state and this part can combine with CO, and (b) that this part has a molar magnetic susceptibility of $12\ 000 \times 10^{-6}$ cgs emu in common with other known ferrohaemoproteins [20–23], which can combine with CO. Thus the magnetic susceptibility of the cytochrome oxidase will be analyzed in accordance with the concept that cytochromes a and a_3 are separate identities [1–3]. On the basis of the assumed susceptibility of $12\ 000 \times 10^{-6}$ cgs emu for a_3 we are able to compute the concentration of cytochrome a_3, and obtain a value of 180 μM, i.e. 35 % of the total haem a. It is further possible to compute a series of values for the molar magnetic susceptibilities of Cu and cytochromes a and a_3 as illustrated by columns 5, 6 and 7 on the basis of the results of Keilin and Hartree [1] and Yonetani [2].

The above arguments do not include any assumption concerning the magnitude of the susceptibility of a''. However, all the three transition metal components of sample 6 are likely to be diamagnetic. From the instrument reading obtained for this sample we calculate, after correction for the diamagnetism of the buffer constituents and for the residual, orbital paramagnetism of the metal atoms [24], a value of -0.63×10^{-6} cgs emu for the mass susceptibility of the cytochrome oxidase. This is of the magnitude to be expected for a lipoprotein with a lipid content of about 20 %, a figure estimated from the partial specific values of 0.81 ml/g. If, however, a'' is paramagnetic with four unpaired electrons, the diamagnetic mass susceptibility of the oxidase would

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* Abbreviations used: Cu'' and Cu': oxidized and reduced copper; a''' and a'': oxidized and reduced cytochrome a; a_3''' and a_3'': oxidized and reduced cytochrome a_3; A''' and A'': oxidized and reduced cytochrome A, representing cytochrome a in a single haemoprotein concept of cytochrome oxidase.

have to be -0.67×10^{-6} cgs emu which is difficult to reconcile with a lipid content of only 20 %. A similar argument cannot be applied to the case that a'' has two unpaired electrons, since the necessary correction of the diamagnetism would be too small. This case must, however, be considered improbable for theoretical reasons [25]. The most reasonable assumption to make is, therefore, that a'' is diamagnetic. For a more precise conclusion on this point it would be necessary to determine more accurately the lipid content and the diamagnetism of cytochrome oxidase. The partial specific volume is of special importance with respect to the latter, since its value has a great influence on the calculated diamagnetism.

The mean susceptibility of the oxidized sample treated with cyanide (sample 2 in Table 1) is $1\,840 \times 10^{-6}$ cgs emu. The ESR absorption data to be described below indicate that practically all the copper in the oxidized samples is present in the paramagnetic cupric form with one unpaired electron. a''' and $a_3'''-CN$ must, therefore, both be low spin forms with one unpaired electron in the iron. A mean value of the susceptibility of haem compounds of this type is $2\,400 \times 10^{-6}$ cgs emu. If this figure is assumed to be valid in the present case the susceptibility of $Cu''-CN$ calculates to $1\,500 \times 10^{-6}$ cgs emu, which is about the maximal value of the known data for cupric compounds [18,19,26].

The increase in susceptibility when going from the cyanide treated oxidized sample to the untreated oxidized sample must be due to the susceptibility difference between $a_3'''-CN$ and a_3''', since a''' does not react with cyanide, and the susceptibility of Cu'' can hardly be larger than the $1\,500 \times 10^{-6}$ cgs emu of $Cu''-CN$. If this value is taken also for the susceptibility of Cu'', the susceptibility of a_3''' is found to be $7\,900 \times 10^{-6}$ cgs emu. The susceptibility of Cu'' might possibly be as much as 250×10^{-6} cgs emu lower, corresponding to $9\,400 \times 10^{-6}$ cgs emu for a_3'''.

From the data in Table 1 it is seen that the magnetic properties of cytochrome a are similar to those of cytochrome c [27]. This is in accord with the similarity in response of the two haemoproteins to CN and CO, and also with their similar function, $i.e.$ transporting electrons on the same level in the electron transporting system [28]. Cytochrome a_3, on the other hand, seems to be different from most other haemoproteins. The reduced form a_3'' is of the high spin type also found for haemoproteins which transport oxygen or have hydroperoxidatic activity. The susceptibility of the oxidized form a_3''', however, is intermediate between the $2\,000$ and $13\,000-15\,000 \times 10^{-6}$ cgs emu usually found for oxidized haemoproteins of the low and high spin forms, respectively. The hydroxides of oxidized myoglobin and haemoglobin have also been found to have similar intermediate susceptibility values [29,30]. These compounds have been shown to be thermally balanced equilibrium mixtures between high and low spin forms [31]. We have recently found that the acid form of leghaemoglobin with a susceptibility of $9\,500 \times 10^{-6}$ at 20°C also must be considered as an equilibrium mixture of this type [22]. The similarity in susceptibility of cytochrome a_3 and leghaemoglobin might be more than coincidental and since the function of the latter is still obscure it might perhaps pay to test it for functional similarities with cytochrome a_3.

The records of ESR spectra of Fig. 1 show that cytochrome oxidase at 77°K gives only a very faint absorption at the magnetic field, where the acid
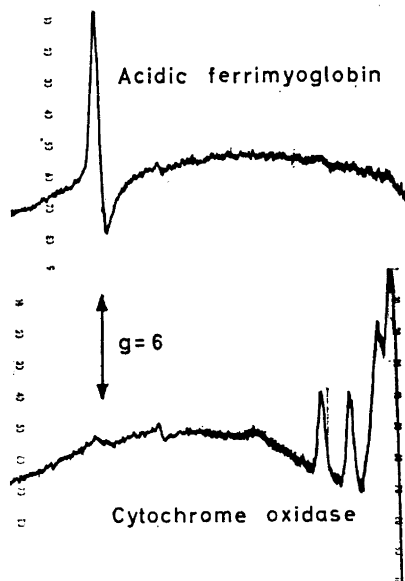
Fig. 1. Derivative records of ESR absorption from acidic ferrimyoglobin (250 μM) and cytochrome oxidase (510 μM in Fe) at 77°K. Field modulation 100 kc, 6 gauss peak to peak. Magnetic field increases from left to right. Microwave power *ca.* 1 mW.

form of ferrimyoglobin has an intense absorption of g-value 6. Derivatives of ferriprotoporphyrin of the high spin type are known to give an ESR absorption with this g-value [32]. The hydroxide of ferrimyoglobin also gives this absorption in aqueous solution at room temperature [33] under which conditions it has been shown to be an equilibrium mixture between high and low spin forms [31]. At 77°K, however, the absorption| is to a large extent abolished, which has been interpreted as due to a shift of the equilibrium in favour of the low spin form [33]. Even if high spin derivatives of ferriporphyrin a are likely to give an ESR absorption of g-value 6, in analogy with high spin ferriprotoporphyrin derivatives, this absorption has not yet been demonstrated experimentally. Due to lack in sensitivity in the present experiments with aqueous solutions it was not possible to test for a possible analogy between a_3''' and ferrimyoglobin hydroxide.

The ESR spectrum of oxidized cytochrome oxidase recorded at 77°K is presented in Fig. 2. The same spectrum, but with a much smaller signal to noise ratio, was obtained at room temperature. The asymmetric shape of record A is typical for cupric compounds of tetragonal structure in glasses [34] and frozen solutions [35], or even in aqueous solutions at room temperature, if the copper is bound to a macromolecule [35]. The integrated intensity of the absorption at room temperature was compared with that of 1 mM cupric ions bound to myoglobin [36]. A ratio of 1:1 of the intensities was found, which indicates that all the copper of the oxidized cytochrome oxidase is in the cupric form.
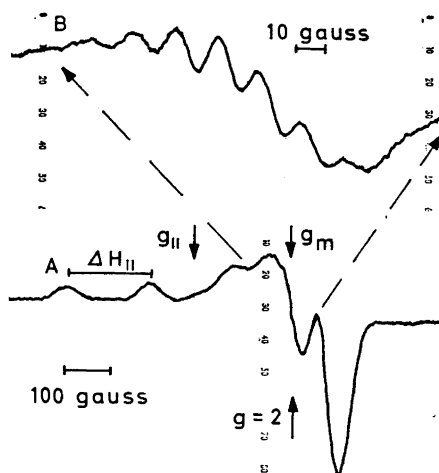
Fig. 2. Derivative records of ESR absorption from copper in cytochrome oxidase (1.025 mM in Cu) at 77°K. Field modulation 100 kc, 12 and 3 gauss peak to peak for A and B, respectively. Magnetic field increases from left to right. Microwave power *ca.* 0.25 mW.

This is in agreement with the result of Takemori [6] and Yonetani [10], who used specific chelating agents to determine the valency of the copper. The ESR absorption completely disappeared after reduction with dithionite, justifying our conclusion that all the copper is in the diamagnetic cuprous form in reduced cytochrome oxidase. Reduction with cytochrome *c* plus ascorbate, or ascorbate alone, likewise removed the absorption to a large extent in the presence or absence of cyanide. This is in accord with the results of Sands and Beinert [7]. Contrary to these authors, however, we have not been able to observe any reduction of the copper in presence of an excess of reduced cytochrome *c* devoid of any additional reducing agent. In one experiment cytochrome oxidase (300 μM in Cu) and cytochrome *c* (3 mM in Fe^{++} and 0.5 mM in Fe^{+++}) were mixed under complete anaerobiosis. After 5 min the sample was frozen and measured in the ESR spectrometer. No reduction of the copper could be observed. Spectroscopic observation of the thawed solution revealed strong absorption bands at 605 and 550 mμ showing that cytochromes *a* and *a$_3$* were reduced and that the excess of reduced cytochrome *c* still persisted. New observations after that the sample had been stored for 20 h at + 3°C gave identical results, both in the ESR spectrometer and in the spectroscope. The cytochrome *c* was immediately oxidized when the sample was exposed to the atmospheric oxygen. These results demonstrate that the copper of the cytochrome oxidase preparation used does not change valence at the same time as the cytochromes *a* and *a$_3$* are reduced by cytochrome *c*.

The g-value in a direction perpendicular to the tetragonal symmetry axis is approximated by g_m, measured at maximum absorption [35]. We obtain $g_m = 2.042$. For the direction parallel to the symmetry axis we find that $g_{||} = 2.185$. $\Delta H_{||}$, the hyperfine spacing due to the copper and valid for the

latter direction, is measured to be 184 gauss. In Fig. 2, record B is a magnification of a portion of curve A. Several, at least 9, additional hyperfine lines are visible and are centered close to g_m. The spacing between the lines is uniform and equals 15.6 gauss.

The shape of the ESR-spectrum is slightly changed when KCN is present, which demonstrates that CN^- in fact is bound to the copper, in agreement with our previous result [11]. The main peak centered close to g_m is decreased and somewhat broadened. g_m and $g_{||}$ are decreased to 2.036 and 2.160, respectively. $\Delta H_{||}$ remains unchanged, but the spacing between the additional hyperfine lines is increased to 17.7 gauss.

Well resolved hyperfine structures due to interaction with nitrogen and hydrogen nuclei in the chelating agent have been observed by Maki and McGarvey for cupric chelates in crystals [37]. The additional hyperfine structure observed here for cytochrome oxidase is no doubt correspondingly caused by an interaction with atoms of the groups of the protein that are coordinated to the copper. This implies a certain degree of delocalisation of the unpaired electron, or, which is equivalent, the associated unpaired hole. The increased splitting when CN^- is bound indicates that the strong field from CN^- enhances this delocalisation. Several cupric complexes have been investigated and similar but not identical hyperfine structures have been found when nitrogen is present among the coordinating atoms but not when there is only oxygen. Imidazole and/or amino groups are hence likely to be involved in the binding of copper to cytochrome oxidase.

The hyperfine spacing $\Delta H_{||} = 184$ gauss corresponds to a splitting constant of 0.019 cm^{-1}, which is of the magnitude ordinarily found for cupric complexes [35]. Malmström and Vänngård recently found the uniquely low value of 0.008 cm^{-1} for two oxidative copper enzymes, laccase and ceruloplasmin, in which copper has been shown to change valence during the enzymatic reaction [35]. They suggested that there is a relation between the enzymatic activity and the high degree of delocalisation of the unpaired hole as evidenced by the small splitting constant. The normal splitting constant of the ESR absorption from the copper of cytochrome oxidase can thus be taken as a further support of our conclusion that this copper does not take part in the enzymatic reaction by changing valence.

From the previous it is seen that the measurement of the magnetic susceptibility of a reduced sample of cytochrome oxidase offers a possibility to estimate the amount of cytochrome a_3 separately. Cytochromes a and a_3 have been demonstrated to contribute 65 and 35 percent of the total a of the present preparation, respectively. The accuracy of the tentative susceptibilities of the derivatives of cytochromes a and a_3 of Table 1 might be improved by measurements on cytochrome oxidase samples devoid of copper. Our experiments with a new copper specific chelating agent, Bathocuproine sulfonate [11] have shown that it should be possible to remove the copper under mild chemical conditions. It has been possible through the use of spectrophotometric and chemical methods [9-11] to obtain evidence that the main portion of the copper of cytochrome oxidase does not take part in the electron transfer of cytochrome oxidase which is in accord with the data presented here. The discrepancy between our results and those of Sands and Beinert [7] concerning the reducibility

of the cytochrome oxidase copper by reduced cytochrome *c* remains to be explained. Since most of the copper probably is an impurity it might be bound differently depending on the method of preparation and hence also respond differently to reducing agents. The observation by Sands and Beinert [7], that added cupric ions were not reduced by ascorbate at the same time as the original copper changes valency, lends some support for this hypothesis.

The previous magnetic titration of cytochrome oxidase [10] was carried out during the reaction of reduced cytochrome oxidase with oxygen in the presence of reducing agents. Hence the observed magnetic susceptibility changes were due to a difference in susceptibility between the reduced and aerobic steady state oxidized preparations while the present results are due to differences between the reduced and completely oxidized preparations. Therefore further experiments are required to interpret the discrepancy between the two results.

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