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EPR SIGNALS FROM CYTOCHROME *c* OXIDASE

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Summary

1 The major EPR signals from native and cytochrome *c*-reduced beef heart cytochrome *c* oxidase (EC 1 9 3 1) are characterized with respect to resonance parameters, number of components and total integrated intensity A mistake in all earlier integrations and simulations of very anisotropic EPR signals is pointed out

2 The so-called Cu^{2+} signal is found to contain at least three components, one "inactive" form and two nearly similar active forms One of the latter forms, corresponding to about 20% of the total EPR detectable Cu, has not been observed earlier and can only be resolved in 35 GHz spectra It is not reduced by cytochrome *c* and is thought to reflect some kind of inhomogeneity in the enzyme preparation The 35 GHz spectrum of the cytochrome *c* reducible component shows a rhombic splitting and can be well simulated with *g*-values 2 18, 2 03 and 1 99 The origin of such a unique type of Cu^{2+} spectrum is discussed

3 The low-spin heme signal in the oxidized enzyme (g = 3 03, 2 21, 1 45) is found to correspond closely to one heme and shows no signs of interaction with other paramagnetic centres

4 The high-spin heme signals appearing in partly reduced oxidase are found to consist of at least three species, one axial and two rhombic types An integration procedure is described that allows the determination of the total integral intensity of high-spin heme EPR signals only by considering the $g = 6$ part of the signals In a titration with ascorbate and cytochrome *c* the maximum intensity of the $g = 6$ species corresponds to 23% of the enzyme concentration

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Introduction

Cytochrome *c* oxidase, (EC 1 9.3.1), the terminal oxidase in the respiratory chain, has been the subject of many physico-chemical studies during the last few years, both in isolated form and in particulate preparations Among the techniques used, the electron paramagnetic resonance (EPR) method has proven to be very powerful, since both the heme groups and the two copper ions supposed to reside in the functional unit of the oxidase may show up in EPR spectra. Recently, for example, Beinert et al [1,2] have presented in two papers a wealth of information on the redox and kinetic properties of the oxidase as studied by EPR

The assignment of the EPR signals to particular components and the quantitative estimate of the signals constitute fundamental problems in the EPR of cytochrome oxidase The present paper contributes to the understanding of these problems. However, it is not our intention to give a systematic study of the properties under various conditions. Rather, we propose to give a careful characterization of the signals in terms of *g*-values, number of components in the signals, signal shapes and absolute intensities from the enzyme in the resting state and partially reduced by cytochrome *c* The findings will form a basis for further studies For example, the unusual *g*-values reported here for the so-called copper signal must be considered in models of the electronic structure of the corresponding electron acceptor Similarly, a correct integration procedure of the high-spin heme signal described in the paper for the first time is important in the assignment of signals to particular cytochromes and in the determination of oxidation-reduction potentials from EPR titrations [3]

Materials and Methods

Enzymes and chemicals

Cytochrome *c* oxidase was prepared from beef heart mitochondria according to the method of Van Buuren [4], but without addition of EDTA As a final step in the preparation procedure, chromatography on a Sephadex G-25 column was performed in order to remove all ammonium sulphate.

The cytochrome *c* used was Sigma type VI from horse heart, further purified by ion exchange chromatography [5].

The concentration of cytochrome *c* was calculated from $\epsilon^{550}_{(red-ox)}$ = 21 1 mM^{-1} cm^{-1} All oxidase concentrations are expressed in terms of a functional unit containing two heme groups and calculated from $\epsilon^{605}_{(red-ox)}$ = 24.0 mM^{-1} cm^{-1} Determination of total iron showed that no extraneous iron was present

Emasol 4130 was obtained from Kao Atlas Co , Ltd , Tokyo All other chemicals were of analytical grade.

Flameless atomic absorption spectrometry with a graphite rod (Perkin-Elmer 403) was used for determinations of the total iron and copper contents of the oxidase. As standards, additions of different amounts of Cu^{2+} and Fe^{3+} to the oxidase solution were made For chemical analysis of Cu, the method of Broman et al [6] with ascorbic acid as reductant, was used

All experiments were performed in 0 1 M phosphate buffer, pH 7.4, containing 0.5% Emasol 4130

Titrations

The anaerobic titrations were made with a system consisting of an EPR tube at the top and two glass bulbs at the bottom One bulb contained the oxidase and the other ferrocytochrome *c* or cytochrome *c* (0 25 times the oxidase concentration) plus varying amounts of ascorbic acid The system was freed from oxygen by repeated evacuation and flushing with oxygen-free nitrogen When anaerobiosis was achieved, the reactants were mixed and the system turned upside down, in order to let the solution pour into the EPR tube

EPR spectra

EPR spectra at 9 GHz were recorded with Varian E-3 or E-9 spectrometers Temperatures between 4 2°K and 40°K were achieved by a helium gas flow system [7] Spectra at 35 GHz were obtained by a Varian V-4503 spectrometer equipped with a helium [8] or nitrogen gas flow system

Integration and simulation of EPR signals

It has recently been pointed out [9] that apparently a mistake has been made in all previous integrations and simulations of EPR signals involving widely separated *g*-values This arises through neglect of the fact that in most spectrometers the field is swept, rather than the frequency For example, the integrated intensity of an isotropic line is proportional to g and not to g^2, a fact that is also of importance in estimates of intensities of so called "half-field" lines from coupled systems [10]

The total intensity of a powder spectrum is approximately proportional to the value of the following expression (ref 9, Eqn. 6)

$$\tfrac{2}{3}\{(g_x^2 + g_y^2 + g_z^2)/3\}^{1/2} + \tfrac{1}{3}(g_x + g_y + g_z)/3$$

which is correct within 1 5% in the range $0\,2 < g_x/g_y \leqslant 1,\ 1 \leqslant g_z/g_y < 8$ If a copper signal is compared to another copper signal used as a standard, the expression given earlier [11] gives the correct value within a few per cent This happens to be the case also if the total intensity of the low-spin heme signal from oxidized cytochrome oxidase (g = 3.03, 2 21, 1 45) is compared to a copper standard However, grossly erroneous results will be obtained in the earlier formulation [11] when a high-spin heme signal is compared to a copper signal

Fig 1 shows two simulated spectra, which are given as illustrations of simulation and integration procedures The *g*-values are 6, 3 and 2 in Fig 1A and 6, 6 and 2 in Fig 1B The line-width is assumed to be anisotropic with the corresponding principle values 20, 30 and 10 mT The line shape in this figure and in the other simulated spectra shown in this paper is Gaussian and the angular dependence of the line width is the same as that of a hyperfine coupling The rationale behind this is that at least part of the line-width might be due to unresolved hyperfine structure. For these simulations it is important to use the correct expression for the intensity [9] An earlier attempt to simulate a spectrum like that in Fig 1B [12] gave a ratio of the peaks at high and low field which deviated by a factor of three Note also how bringing two *g*-values together in Fig 1B raises the amplitude of the signal as compared to Fig 1A,

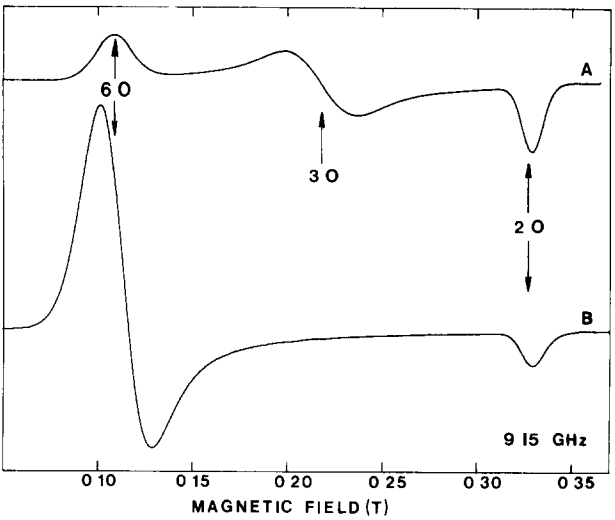

Fig 1 Simulated EPR spectra at microwave frequency, 9 15 GHz and g-values 6 0, 3 0, 2 0 (A) and 6 0, 6 0, 2 0 (B) For both spectra the line-widths are 20, 30 and 10 mT at the corresponding g-values As for the simulated spectra shown in Figs 3—5, a Gaussian line shape is used with the angular dependence of the line-width taken as that of a first order hyperfine splitting

although the two spectra correspond to the same number of metal ions. The peaks at highest field do not exactly coincide with the corresponding g-value This is an effect of the particular choice of the angular dependence of the width (see above) Other simulations have shown that if the width was caused by an independent spread in the principal g-values, the shift would be much less pronounced. For the spectra in Figs. 3—5 the width is so small compared to the total spread of the spectra that no appreciable shift appears in any of the two models for the angular dependence

It is obvious that in cases where the whole shape of an experimental spectrum is not available, e g due to overlap of many spectra, the intensity can still be obtained through a comparison to a simulated spectrum, provided the resonance parameters are known However, methods can be developed which in some cases allow determinations of spin concentrations without reference to simulated spectra The procedures for obtaining the total intensity from the isolated "absorption-type" peaks at high or low fields in Fig 1A or from the peak at high field in Fig. 1B are described in detail in ref. 9 and will not be repeated here

For high-spin heme signals of cytochrome oxidase it is important to determine the spin concentrations from the part of a spectrum where two g-values coincide, such as at low fields in Fig. 1B, without having access to the small peak at high fields Earlier, Beinert et al [13,14] used myoglobin as a reference in this case and applied a formula which related the double integral over a certain part of the spectrum to the peak-to-peak width of the low-field signal This formula was obtained from some spectra simulated under the assumption of axial symmetry Now, many of the signals of interest show rhombic splitting of this line, which however is not large enough to make the procedure for "absorption-type" peaks applicable An alternative method is described below

which is based on the fact that, within certain limits, the double integral of the $g = 6$ peak is independent of the line-width and a rhombic splitting Thus, at a microwave frequency of 9100—9500 MHz, the double integral of the absorption derivative with the lower integration limit taken below the low-field end of the spectrum, and the upper limit at a field corresponding to an apparent g-value of 4 67, yields 47—49% of the total double integral This is valid for widths less than 6 mT, $g_z = 2\,0$, $1/2(g_x + g_y) = 5\,85$—$5\,95$ and rhombic splittings $(g_x - g_y)$ less than 1 0 The $g = 6$ signals we have observed in cytochrome oxidase all fall within these limits Note that the method of first moment, sometimes used to obtain the double integral of whole spectra, should not be applied in this connection The signal amplitude at the upper limit equals 350—450 times the double integral up to the same limit multiplied by the square of the integration interval in units of tesla This relation can be used to check the way the baseline has been drawn

Results and Discussion

General characteristics of the EPR signals

The EPR signals from metal ions in cytochrome oxidase are due to either divalent copper or trivalent iron

Cu^{2+} has a d^9 configuration with $S = 1/2$ It prefers a tetragonal or slightly distorted tetragonal ligand field which gives g-values in the range 2 0—2 4. It is often best studied at temperatures around that of liquid nitrogen (77°K) or higher Lower temperatures usually add no new information, but microwave power saturation of the signal may be difficult to avoid in this case In the EPR spectrum of cytochrome oxidase, Fig 2A, the lines at $g = 2\,18$ and $g = 2\,00$ are supposed to arise from Cu^{2+} (see Discussion below) At higher temperatures other lines seen in Fig 2 are broadened and only the Cu^{2+} signal can be observed easily. Note that in Fig 2 the Cu^{2+} signal is strongly saturated but still has the largest apparent amplitude

Fe^{3+} with a d^5 configuration can have $S = 5/2$ or $S = 1/2$, called respectively high- and low-spin, depending on the strength of the ligand field High-spin heme Fe^{3+} has an isolated Kramers' doublet lowest with excited states usually about 10—30 cm^{-1} higher in energy EPR absorption is only observed from the lowest doublet with one g-value, g_z, very near 2 00 and the other two, g_x and g_y, around 6 0 In a perfectly tetragonal ligand field $g_x = g_y$, but there often is a small rhombic distortion giving $g_{x,y}$ in the range 6.5—5 3 The peaks at $g = 6$ always have much larger apparent amplitudes than the $g = 2$ peak (cf Fig 1) and can be observed even at 77°K, although broader Generally, however, high-spin heme signals are best studied at lower temperatures because of increased resolution In cytochrome oxidase (Fig 2) only the lines near $g = 6$ can be observed, because there are always some other resonance features masking the weak $g = 2$ absorption

Low-spin heme Fe^{3+} has one unpaired electron in a t_{2g} orbital The ligand field has a symmetry typically lower than the axial symmetry, and a rhombic type of EPR spectrum is observed with three g-values ranging from 0 8 to 3.5 EPR detectability is essentially the same as for high-spin heme Fe^{3+} although

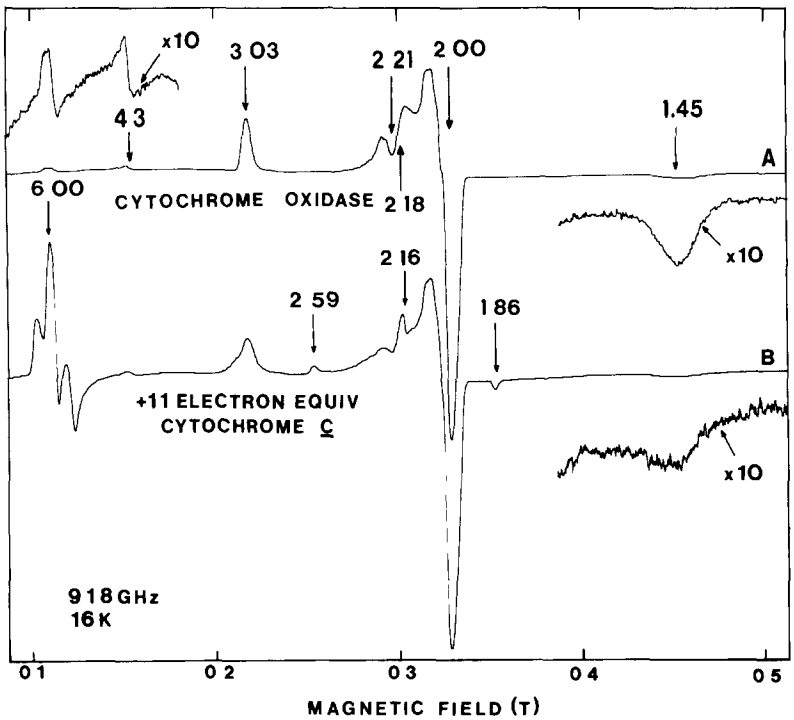

Fig 2 EPR spectra of cytochrome *c* oxidase recorded at microwave frequency, 9 18 GHz, temperature, 16°K and microwave power, 2 mW Trace A, isolated enzyme, 0 23 mM, in 0 1 M phosphate buffer, pH 7 4, containing 0 5% Emasol 4130, trace B, enzyme partially reduced with 1 1 electron equivalents cytochrome *c*, the gain is increased to compensate for the dilution of the enzyme The so-called Cu^{2+} signal with *g*-values 2 18 and 2 00 and the low-spin Fe^{3+} signal with *g*-values 2 59, 2 16 and 1 86 appearing on reduction are strongly saturated under the chosen instrumental conditions

low-spin signals are usually more easily saturated In Fig 2 the peaks at $g = 3.03$, 2.21 and 1 45 arise from one low-spin species, whereas the peaks at $g = 2\ 59$, 2.16 and 1.86 are due to another low-spin species Cytochrome *c* also has a low-spin heme Fe^{3+} with *g*-values 3 0, 2.2 and 1.2 [15]. The peaks at the two latter *g*-values are very broad and not observed in Fig. 2B The broadening of the $g = 3\ 03$ peak in Fig. 2B is, however, due to oxidized cytochrome *c*

The signal at $g = 4\ 3$ in Fig 2 is attributed to non-heme high-spin Fe^{3+} in a ligand field of very low symmetry, usually called "rhombic iron". Such a signal is observed from almost all biological materials and is supposed to be due to extraneous iron or denatured material As this type of signal is rather narrow, an observable peak can be obtained even from negligible amounts of Fe^{3+} The $g = 4.3$ signal seen in Fig. 2 is supposed to belong to the latter category and, accordingly, not further discussed in this paper.

The Cu^{2+} signal

The total intensity of the Cu^{2+} signal at 77°K in native cytochrome oxidase was found to correspond to about 100% of the enzyme concentration, i e. 1 Cu^{2+}/functional unit The copper signal always consists of at least two species [16], one major part, previously called active or functional copper, with *g*-values 2.18 and 2.00 and a minor species resembling organic Cu^{2+}-complexes

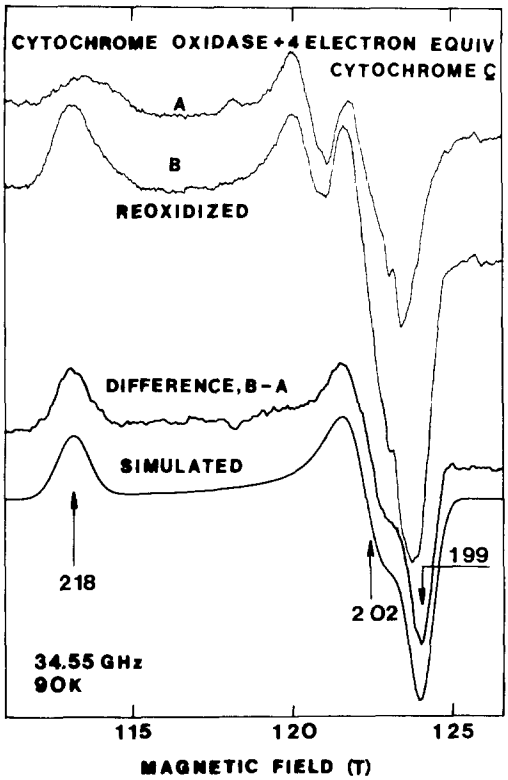

Fig 3 EPR spectra of the $g = 2$ region of cytochrome *c* oxidase Microwave frequency, 34 55 GHz, temperature, 90°K, microwave power, approx 5 mW Trace A, enzyme, 0 24 mM, reduced with 4 electron equivalents cytochrome *c*, trace B, reoxidized by air for about 5 min, third trace from top, difference spectrum, B-A, lower trace, simulated spectrum with *g*-values (2 182, 2 019, 1 990) and line-widths (10, 11 5, 7 5) mT

with nitrogen ligands The intensity of the latter species, called "inactive" or EPR-detectable extraneous copper, varies for different preparations but in our preparations most often corresponds to about 25% of the total EPR-detectable Cu^{2+}

In order to obtain an EPR spectrum of the cytochrome *c*-reducible Cu^{2+} the experiment shown in Fig 3 was performed Spectra were recorded at 35 GHz because of increased resolution Trace A shows the spectrum of cytochrome oxidase anaerobically mixed with four electron equivalents of reduced cytochrome *c* for about 20 min, while trace B shows the spectrum of reoxidized enzyme The line at 1 20 T is due to "inactive" copper because treatment with CN^-, known to reduce the "inactive" Cu^{2+} signal [16], removes this line Most of the remaining features in trace A are due to a type of cytochrome oxidase Cu^{2+} apparently not reduced by cytochrome *c* under the given conditions. This form can also be seen in trace B making the $g = 2$ 18 peak at 1 13 T asymmetric We estimate that it corresponds to about 20% of the total EPR-detectable Cu^{2+} and probably reflects an inhomogeneity in the enzyme preparation. As this type of inhomogeneity in the Cu^{2+} signal can hardly be detected in 9 GHz spectra we do not know if it is present in all our preparations or in preparations

used in other laboratories However, the two preparations which we have recorded at 35 GHz show the same kind of asymmetry in the peak at 1.13 T This asymmetry is not removed by CN^- treatment

In the difference spectrum shown in Fig. 3, the "inactive" Cu^{2+} and the active type which is not reduced by cytochrome *c* are eliminated. The bottom trace in Fig 3 shows a computer simulated spectrum of the cytochrome *c*-reducible component The EPR parameters are very unusual for a Cu^{2+} spectrum. Thus, the simulation is made without any copper hyperfine splittings and if such splittings are included, the fitting with the experimental spectrum is not improved Furthermore, one *g*-value must be significantly less than the free-electron *g*-value 2 00. This is hardly compatible with a "normal" type of Cu^{2+} coordination which can be described as at least having essentially tetragonal symmetry. However, it is known that very unusual *g*-values and hyperfine splittings can be obtained from Cu^{2+} forced into specific geometries [17,18] Merely the combination of very small *g*-value and hyperfine splitting in the parallel direction places the cytochrome oxidase Cu^{2+} EPR spectrum in a unique position among Cu^{2+} signals [19] The microwave saturation behaviour is also unique as this EPR signal is much more difficult to saturate than most other Cu^{2+} complexes including protein-bound Cu^{2+}

The very unusual features of the Cu^{2+} signal have caused some doubt as to whether it is due to copper at all but instead rather could arise from low-spin Fe^{3+}, i e. the second heme in cytochrome oxidase [3]. Although it is difficult to find any definite evidence for this signal arising from copper, recent experiments with carbon monoxide seem to exclude the low-spin Fe^{3+} alternative [2] Thus, it was found that cytochrome oxidase reduced in the presence of CO and reoxidized by ferricyanide showed the same low-spin heme and Cu^{2+} EPR signals as the oxidized protein although CO apparently still was bound to one reduced heme It has also been suggested [20] that the signal is due instead to a kind of sulphur radical However, in order to get a radical *g*-value as high as 2 18 an improbably high symmetry is probably needed. Furthermore, a radical model would create a potential fifth electron acceptor in the oxidase, which is not in keeping with titration studies [1]

At this stage it is probably not necessary to invoke interaction with other paramagnetic centres to explain the unique Cu^{2+} EPR signal Instead, there are several arguments against interaction models Titration studies [1] have shown that the signal is not due to a two-electron acceptor. Furthermore, potentiometric titrations [21] indicate that the EPR-undetectable copper is reduced in the presence of CO under conditions where the signal is present [1] This seemingly excludes Cu-Cu interaction. An interaction involving the low-spin heme (g = 3 0, 2 2, 1 4) is also unlikely since its EPR signal shows no indications of interaction effects (see below). Thus, we still believe that the so-called Cu^{2+} signal is in fact due to Cu^{2+} situated in a unique type of protein metal binding site

At present it is not understood why both Beinert's group [14] and ours find only 0 7—0.8 Cu^{2+}/functional unit after correction for the "inactive" form In the present work there is still another problem because the particular preparation used for the detailed copper study had a total copper content of 2.7—2 8 Cu/functional unit as determined both by atomic absorption and chemical

analyses This high value is probably due, at least partly, to the omission of EDTA in the preparation procedure

The low-spin heme signal

The low-spin heme Fe^{3+} signal from oxidized enzyme is shown in an expanded scale in Fig 4 As seen in the lower tracing, this signal can be simulated quite well using g-values 3 034, 2.213 and 1 446. The g-values and shape are typical for a non-interacting low-spin heme Applying the procedure described in ref [9] the intensity of the signal was compared with a Cu^{2+} standard sample. Both the g = 3 03 and the g = 1 45 peaks were utilized and the result is that this low-spin signal corresponds closely to 100% of the enzyme concentration. The corresponding figure reported by Hartzell and Beinert [14] was only 70% The reason for this discrepancy is currently under investigation in the two laboratories (see ref 1 for further discussion)

The g = 3 03 peak is always somewhat asymmetric, showing a slight variation from preparation to preparation also as reported earlier [14] Although we have not made any detailed study, this asymmetry may be related to the kind of inhomogeneity discussed above for the Cu^{2+} signal On the other hand, we cannot observe in our reduction experiments any clear splitting of the g = 3 03 peak into two components [14] With some reservation for the observed inhomogeneities, we therefore conclude that the low-spin signal in oxidized cytochrome oxidase corresponds to one heme showing no sign of interaction with other paramagnetic centres. The proposed symmetrical model, in

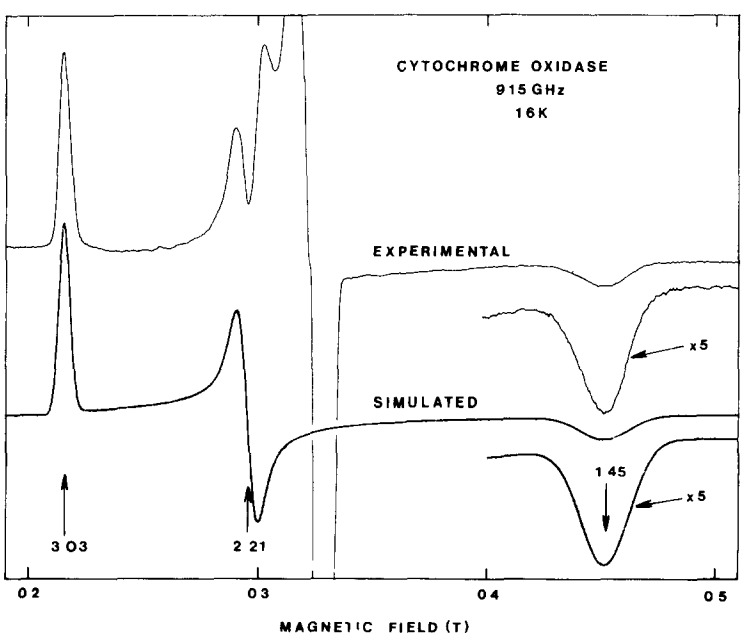

Fig 4 Experimental EPR spectrum of cytochrome *c* oxidase showing the low-spin heme signal in detail (upper trace) Sample and instrumental conditions as in trace A of Fig 2 except for a microwave frequency of 9 15 GHz Lower trace is a simulated spectrum with g-values (3 034, 2 213, 1 446) and line-widths (4 8, 7 0, 20 5) mT

which both heme groups are identical and both therefore should contribute to the low-spin signal [22], is hardly compatible with this conclusion

The high-spin heme signal

A small signal at $g = 6$ is always seen in the oxidized enzyme (Fig. 2A) (cf. ref. [14]). At least part of it is probably due to autoreduction because we have found that addition of ferricyanide decreases the signal. In our reductive titrations with ascorbate and cytochrome *c*, this residual signal was first seen to increase, but then distinctly new, much stronger $g = 6$ species appear. Fig 5 shows the $g = 6$ region after reduction with cytochrome *c*. It is evident that this signal must be composed of at least two species, one nearly axial and one more rhombic [cf ref. 14]. In fact, there must be at least two rhombic types which can be more easily seen in the 35 GHz spectrum shown in Fig. 6. Simulation with one nearly axial and two rhombic species also gives a reasonable fit to the experimental spectrum at 9 GHz (Fig. 5). Most probably, an even better fit can be obtained by adjustment of line-widths and relative weights of the components However, the sum of the relative weights of the rhombic components compared to that of the axial component cannot be changed very much from that used for the simulated spectrum in Fig 5 Note, that it is our minor rhombic species that most closely resembles the rhombic $g = 6$ species observed by Hartzell and Beinert in samples reduced by cytochrome *c* [14].

The total intensity of the $g = 6$ species was calculated from the truncated

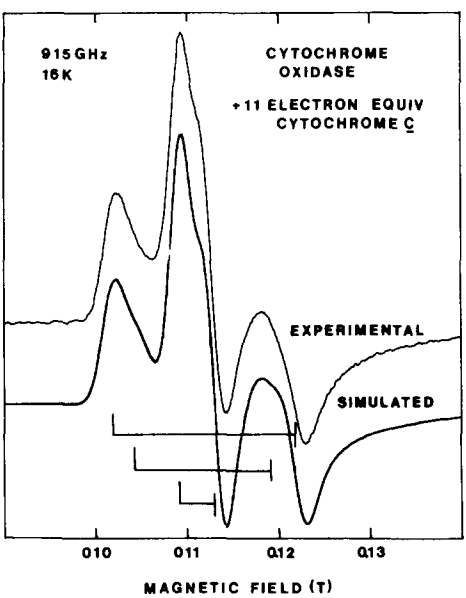

Fig 5 Experimental EPR spectrum of partially reduced cytochrome *c* oxidase showing the region around $g = 6$ in detail (upper trace) Sample and instrumental conditions as in trace B of Fig 2, except for a microwave frequency of 9 15 GHz Lower trace is a simulated spectrum obtained by adding three different components in a suitable proportion The *g*-values, line-widths and relative weights of these components are (6 421, 5 371, 2 0), (2 4, 2 4, 2 4) mT, 1 0, (6 270, 5 486, 2 0), (3 1, 3 1, 3 1) mT, 0 7 and (5 988, 5 787, 2 0), (2 1, 2 1, 2 1) mT, 0 8 respectively The *g*-values of the components are shown by the schematic stick spectra in which lines above and across the base line indicate "absorption" and "derivative" type of lines, respectively

second integral by the procedure described in Materials and Methods The maximum concentration, observed after addition of about three electron equivalents of ascorbate, was found to correspond to about 23% of the enzyme concentration. For this calculation, we have assumed that the zero field splitting is the same as in whale metmyoglobin (D = 10 5 cm^{-1}) A separate experiment confirmed this earlier finding [14] It was found that the sum of all heme signals corresponds to one or less than one heme during the whole titration [cf ref 14]

A correct estimate of intensities is important in interpretations of potentiometric titrations of the g = 6 signal [3] where bell-shaped curves of intensities vs potential have been observed [23] For example, if the maximum of the curve corresponds to 23% of the fully oxidized one-electron acceptor, the upper and lower inflexion points could give potentials that are up to 50 mV too high and low respectively

Other signals

A signal with g-values 2 59, 2 16 and 1.86 is observed on partial reduction of the enzyme (Fig 2B) Such a signal has been observed earlier [13], but in contrast to Beinert et al [14] we never observed it in the protein as prepared The g-values are typical for a low-spin heme Fe^{3+} but other features of this signal are somewhat unusual Thus, the line-widths of the peaks are very narrow and the saturation behaviour is very much the same as for the oxidase Cu^{2+} signal In our titration with ascorbate and cytochrome c the maximum intensity of this signal, after addition of about two electron equivalents of ascorbate, was found to correspond to about 7% of the oxidase concentration This figure is not very helpful in assigning the signal to a specific heme in the enzyme Before this signal disappears on further reduction, a clear splitting into two species is observed in the g = 2 59 peak This was also observed by Hartzell and Beinert [14]

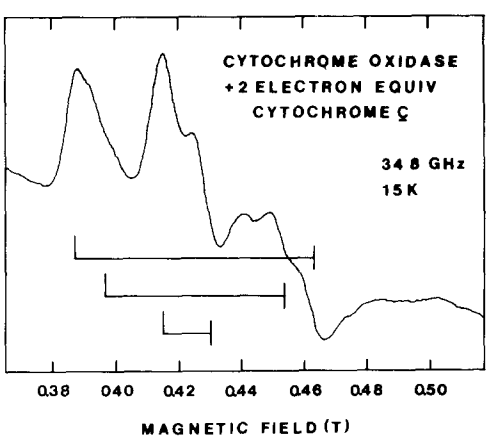

Fig 6 EPR spectrum of 0 1 mM cytochrome c oxidase partially reduced with 2 electron equivalents cytochrome c showing the region around g = 6 Microwave frequency, 34 8 GHz, temperature, 15°K, microwave power, approx 5 mW The stick spectra show the g-values of the three components used in the simulation at 9 15 GHz (Fig 5)

Some preparations and titration experiments gave additional signals, apparently best described as low-spin heme Fe^{3+} Because of their variability we have not made any systematic study of these additional signals and they are not further discussed here. Finally, Yonetani (personal communication) has observed a signal at $g = 12$, and a similar signal is also present in our preparations.

Concluding remarks

This paper is not a systematic study of all EPR signals found in cytochrome oxidase That such a study would demand a tremendous amount of work is illustrated by the number of different signals we have found in our preparations Thus, there are at least 10 EPR signals, most of which were also observed in other laboratories. It is possible that some of the signals which we have not discussed in this paper are of great relevance However, we think that many of these varying signals are due to inhomogeneities in the enzyme preparations. Especially inhomogeneities in molecular size, i e. polymers of different size, might be an important source of the problems with the EPR signals However, one should be aware that a system with four potentially EPR-active electron acceptors can give a large number of EPR signals If interactions are allowed, this number can indeed greatly exceed the observed number of signals

Acknowledgements

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