Properties of the Copper Associated with Cytochrome Oxidase as Studied by Paramagnetic Resonance Spectroscopy*

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There appears to be general agreement that cytochrome oxidase preparations obtained by various procedures contain amounts of copper in the range of the heme content of this enzyme (1–9). Various workers disagree, however, on the significance of this finding (6–10). Whereas several investigators hold the view that the copper associated with cytochrome oxidase has properties that would qualify it as a functional oxidation-reduction component of the oxidase (2, 4, 8, 9), others tend to consider this copper as an impurity (6, 7, 10). Experiments have been reported that support both points of view. A final decision, however, cannot be made on the basis of presently available information. Experiments that would demonstrate that copper undergoes oxidation-reduction at a rate commensurate with the activity of the oxidase are technically rather difficult.

This paper presents the results of work on reactions and properties of the copper of cytochrome oxidase, which may be studied by the technique of paramagnetic resonance spectroscopy. With this technique, it is possible to probe into the immediate environment of the copper atom and obtain information on its valency and binding state. Although we must admit that we are unable to determine the exact environment of the copper from paramagnetic resonance spectra obtained from materials as complex as cytochrome oxidase, it is possible, as will be shown below, to observe distinct changes of paramagnetic resonance spectra under certain conditions, which can be evaluated on a largely empirical basis.

We have studied the interaction of the copper of cytochrome oxidase with reducing substrates, chemical reducing agents, oxygen, inhibitors, and chelating agents for copper. This work forms a basis for future studies on the early kinetics of oxidation-reduction of the components of the oxidase under various conditions, which, we hope, will provide an answer to the question of the function of the copper in the enzyme.

EXPERIMENTAL PROCEDURE

Preparations—Cytochrome oxidase was prepared and assayed as described previously (5). In this type of preparation, a certain amount of deoxycholate (approximately 1 mg per mg of protein) remains bound to the enzyme. At high protein concentrations, such as are used in EPR studies, the concentration of deoxycholate reaches an inhibitory level.

The cytochrome c used was a commercial preparation (Sigma type III). Ascorbate solutions were freshly prepared and kept in ice at pH 4. The pH was adjusted to 6.5 immediately before use.

Methods

Chemical—Protein was determined by the biuret method. In routine work no correction was applied for the intrinsic color of the enzyme preparations. When the heme component was destroyed by hydrogen peroxide treatment (7), the actual protein values were found to be approximately 6% lower than the uncorrected values. Copper and heme were determined as previously described (5).

Spectroscopy—EPR spectroscopy was carried out with a standard Varian V 4500-10A spectrometer equipped with a 100-kc field modulation unit and an accessory for work at variable temperature. The samples and additions were pipetted into the sample tubes with calibrated polyethylene hoses of different width, which were fitted to needles and syringes. For the samples, quartz tubes of 0.5-mm wall thickness and 3-mm inner diameter were used. The tubes were matched for quantitative work. The samples were frozen in liquid nitrogen and were examined at $-176^\circ$. The field was scanned at different modulation amplitudes (4 to 20 gauss) to avoid loss of detail by overmodulation. The records are presented in the form of the first derivative of the absorption curve. For quantitative evaluation, the derivative curve was integrated, and the area under the absorption curve was compared with that of a copper standard. Samples of cytochrome oxidase were also wet ashed with sulfuric acid and hydrogen peroxide, and the resultant digests in concentrated sulfuric acid were compared with identically treated copper sulfate standards.

The location of an absorption band in the EPR spectrum is determined by its g value (cf. (11)). In metal complexes with anisotropic, axially symmetric, crystalline field, two g values are observable, $g_1$ and $g_2$, which refer to the orientation of the external magnetic field with respect to the symmetry axis of the crystalline electric field. The position labeled $g = 2.00$ in the figures corresponds to the location of the relatively sharp absorption band of free electrons, as they are found in free radicals. This position is used as a reference marker, as it is readily re-

* The abbreviations used are: EPR, electron paramagnetic resonance; BCS, bathocuproinedisulfonate.

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cause signal intensity does not follow a simple relationship to concentration, which is imposed by conventional practices of enzyme chemistry. The most stringent requirements are met that necessitate a departure from conventional conditions. In such concentrated samples, inhibitory effects of substances may show up that go unnoticed under conventional conditions. An example with which we were faced in the present work is the inhibition by bile salts that are bound to the enzyme. Another example would be the possible inhibition by substrate if one tries to supply it in saturating quantities. On the other hand, inhibitors, when added at a concentration considered optimal in conventional work, may not show much effect when the protein concentration is high.

Spectrophotometric measurements were carried out with a Beckman spectrophotometer, model DU, equipped with a photomultiplier. Cuvettes of 1-mm light path and 0.1-ml volume were used, so that samples could be studied at the same concentration as was required for EPR measurements. Further details of combined EPR and optical measurements are given in the legend to Fig. 5.

INTERPRETATION OF SPECTRA

An EPR spectrum of Cu²⁺ does not exist because of the diamagnetic state of the ion. The EPR spectrum of the cupric ion, Cu²⁺, does exist, however, and is influenced by several factors reflecting the physical environment of the ion. The ground state is described most closely by a 1s⁶ 2s² 2p⁶ 3s² 3p⁴ 3d⁹ configuration with small amounts of 3s 3p⁴ 3d⁸ ns, etc. contributing. This allows the ion to have an isotropic hyperfine interaction between the electron spin and the nuclear magnetic moments, giving rise, in general, to a superposition of four different sets of spectra, one each from the set of copper isotopes having their nuclei oriented in one of the four possible directions with respect to the electron spin (both copper isotopes, Cu⁶⁷ and Cu⁶⁵, have a nuclear angular momentum of 3/2 and have roughly the same interaction with the electron). The remaining structure of the spectrum is caused by a spin-orbit interaction mixing in various amounts of orbital angular momentum, the latter being quenched in first order by the electrostatic fields imposed on the ion by its environment.

As examples, let us consider the spectra displayed in Fig. 1. Since Cu²⁺ spectra vary with the type of ligands present, we thought that it would be most useful for purposes of comparison to show the spectra of Cu²⁺ in the very medium in which our cytochrome oxidase preparations are dissolved. Fig. 1A, for instance, presents the spectrum of copper sulfate in a sucrose solution containing deoxycholate and lipid, a mixture that resembles that of copper sulfate in ethylenediaminetetraacetate (EDTA) solution and also that from Cu²⁺ in a soda lime silica glass as explained in detail by Sands (12). The parameters of

![EPR spectra of copper sulfate with various additions.](image)
interest for the spectrum of Fig. 1A are $g_1 = 2.29(3)$, $g_m = 2.07(0)$, and $\Lambda_1 = 0.016$ cm$^{-1}$, where $\Lambda$ represents the hyperfine coupling constant. The spectrum of Fig. 1B is of the same general type. The parameters there are $g_1 = 2.36(1)$, $g_2 = 2.06(0)$, $\Lambda_1 = 0.016$ cm$^{-1}$, $A_1 = 0.0024$ cm$^{-1}$, and $\kappa = 0.24$, where $\kappa$ is taken from Sands' paper (12) to represent the amount of s state.

The spectra obtained from the enzyme (Fig. 2, A to D) indicate that still another interaction is operating there; namely, that of exchange (13, 14). This is evidenced by the lack of nuclear hyperfine structure in those spectra and is of considerable interest in that it indicates that at least 2 copper ions are in close proximity to one another in the enzyme in such a way that they experience a relatively large exchange interaction with one another. The measured $g$ values are consistent with such an interpretation. However, it may not be ascertained at present whether all of the ions are in the cupric or whether some are in the cuprous state. For Fig. 2A, which represents the cytochrome oxidase spectrum of submitochondrial particles, and for Fig. 2D, which shows the spectrum of the purified, cyanide-dialyzed enzyme, the $g$ values are $g_2 = 2.17$ and $g_m = 2.03$.

Further support for our conclusion that an exchange interaction between at least 2 copper ions in the enzyme takes place stems from integrations of the spectra. When a double integration of spectra of the type of Fig. 2D is carried out and the area under the absorption curve is compared with that of a standard copper sulfate or copper nitrate-EDTA (15) solution, consistently only approximately 35% of the copper found in the enzyme by chemical analysis is accounted for. When, however, the same enzyme is ashed and the solution of the resulting inorganic material is compared with a correspondingly treated standard, which contains the amount of copper expected in the enzyme according to chemical analysis, and 15.7 mmoles of CuSO$_4$ (B) were ashed as described under "Methods." Of the final solution, 0.05 ml in 0.20 ml of concentrated sulfuric acid was used for measurements at $-176^\circ$.

Theoretical considerations predict$^3$ (cf. (16)) for the case of exchange interaction that double integration of such a signal may result in an area smaller than that found for a signal from separated ions. The diminution in intensity depends upon the relative magnitudes of the exchange coupling and the hyperfine coupling. From the observation that the hyperfine coupling is completely averaged, whereas the $g$ value anisotropy is not, the exchange interaction is estimated to be approximately 0.02 cm$^{-1}$. From observations on isolated cupric ions, the hyperfine coupling is also of the same order of magnitude. These values for the interactions would produce a diminution in intensity, the exact value of which is very difficult to compute. We estimate that this diminution could be as marked as that observed and that minimally the integrated intensity should be one-third of that expected in the case of no interaction. For these reasons it is not possible to draw unambiguous conclusions as to the valence state of the copper ions involved. On the basis of our EPR studies, we are inclined to think that an interaction leading to the maximal possible diminution is unlikely and that in fact the copper ions may occur in pairs, one in the cupric and one in the cuprous form.$^4$ This, admittedly, is not in agreement with the

$^3$ R. H. Sands and H. Beinert, manuscript in preparation.

$^4$ An attempt is being made by one of the authors (R. H. Sands) to compute accurately the diminution from theoretical considerations. If this effort is successful, the result will be published at a
results of chemical analysis (9), but it remains uncertain, whether the destructive chemical analysis can be completely relied upon in the situation encountered here.

An additional point deserves mentioning here. If it is assumed for the purposes of this discussion that copper is indeed the constituent of cytochrome oxidase that interacts directly with oxygen, it is difficult to visualize an interaction of a single copper atom with the oxygen molecule. Our finding of a strong interaction of 2 vicinal copper atoms in the enzyme may here be of specific significance.

Fig. 2B shows the EPR spectrum typical of our cytochrome oxidase preparations as they are obtained by the routine isolation procedure (5). The spectrum of Fig. 4A is identical with that of Fig. 2B except for a 3.2 times faster rate of scan, which makes the widely spaced fine structure more apparent.

This type of spectrum probably represents a superposition of two spectra, one from the copper of the active enzyme and one from delocalized copper of a denatured form or of extraneous copper bound in different loci. This is indicated by the fact that the hyperfine structure is on only one peak and the remainder of the spectrum is characteristic of a strong exchange interaction, which would wash out any hyperfine structure if the latter were due to the same system. The two more distant components of the fine structure (cf. Fig. 4A) are associated with an underlying spectrum from the damaged enzyme or extraneous copper bound to the enzyme. They are practically absent in the enzyme that had been dialyzed against a cyanide solution (2D and 4B). Cyanide treatment is known to decrease the copper to heme ratio of the oxidase to a value very close to unity (9) without seriously affecting the enzymatic activity. These observations suggest that some of the minor features of the EPR spectrum of the oxidase of Fig. 2B and Fig. 4A are due to a small quantity of contaminating extraneous Cu²⁺ ions, which are removed by cyanide. This applies specifically to the additional fine structure that is seen in several of the presented spectra (Figs. 2B, 4A, 5B, 9A) within approximately 100 gauss of the g = 2.00 marker toward lower field. This narrowly spaced structure (≈16 gauss between lines) is thought to belong to a set of 9 lines, which indicates coordination of Cu²⁺ with nitrogen (15, 19).

Another example is hemocyanin. Since here the copper ions are vicinal (18), it is of interest to note that there is a large exchange interaction and a corresponding reduction in integrated intensity for the copper spectra of this protein in the snail, erab, and lobster (P. DiLavore and R. H. Sands, unpublished observations). Whereas the relative magnitudes of g and \( \xi \) are reversed in these hemocyanins compared with those of cytochrome oxidase and ceruloplasmin, this observation is consistent with theoretical interpretation. Similar findings are reported in the review article by Bowers and Owen (14).

We conclude, then, that the EPR spectrum of cytochrome oxidase in its natural state is better represented by Fig. 2, A or D, and Fig. 4B than by spectra similar to that of Fig. 2B.

Cyanide was removed by subsequent dialysis against 0.01 M Tris-acetate of pH 8.0.
It is of interest to note here that the EPR spectrum of cytochrome oxidase published by Ehrenberg and Yonetani (10) is definitely of the type resembling our Fig. 2B and Fig. 4A and is more closely similar to the spectra that we obtain from what we call damaged enzyme (Fig. 7, B to D, Fig. 9, B to D) or enzyme after addition of extraneous copper (Fig. 6B, 7E). We consider it possible that some treatment, such as the long dialysis (72 hours) of Yonetani's enzyme before spectral analyses—a procedure we find detrimental to our enzyme—may have brought about changes in the environment of the copper, such as substitution of ligands and lifting of the interaction of the vicinal copper atoms.

Reduction by Substrate—It was observed previously by Sands and Beinert (20) that the Cu$^{2+}$ signal of cytochrome oxidase decreases in intensity to approximately 20% of its original value when substrate is added to the enzyme. The assumption that disappearance of the Cu$^{2+}$ signal indicated reduction to Cu$^+$ was based on the fact that the disappearance was reversible and that signal intensity responded readily to oxidizing and reducing conditions. In addition, the Cu$^{2+}$ signal is known to be variable in shape, depending on its environment, but it is not known to be very readily abolished under any condition other than reduction.

The observations of Sands and Beinert were substantiated in the present work, and an attempt was made to correlate the rate and extent of the reduction of the copper with those of the heme component. Experiments of the type described in Fig. 5, A and B were therefore carried out in which the rate of reaction was followed spectrophotometrically at the same time as reduction of the copper was measured by EPR on identically treated aliquots of the same enzyme preparation. A description of the experimental procedure is found in the legend. It is evident that the oxidation state of the copper qualitatively parallels that of the heme, although it appears that the copper is more extensively reduced during the steady state. Chemical analyses by Griffiths and Wharton (9) also showed that the oxidation states of the heme and copper components of the oxidase are parallel during reduction of the enzyme. It was necessary in the experiments described here to slow the rate of reaction to a level suitable for the measurements. This was accomplished by limiting the amount of cytochrome c. In addition, the relatively high deoxycholate level, which prevails in the concentrated enzyme solutions used, strongly inhibits the reduction of cytochrome oxidase by cytochrome c. Although these conditions lowered the reaction rate to a convenient range, the slowness of interaction of the otherwise highly active enzyme seriously limits the conclusions that may be drawn from these experiments. It should be pointed out, however, that the experiments of Griffiths and Wharton (9) in which the oxidation state of the copper was assessed chemically, and the results of which agree with our EPR data, were performed at a protein concentration of one tenth of that used in the present work.

Most of the experiments on the reduction of the oxidase by substrate were carried out aerobically. It was observed that in the sample tubes used for EPR measurements, a small top layer always remained in the oxidized state. This would partly account for the observation that approximately 15% of the copper in the enzyme remains in the cupric form even after prolonged exposure to substrate. More rapid and complete reduction was observed anaerobically and also in the presence of cyanide (0.02 M) or azide (0.02 M).

Reduction by Chemical Agents—In the presence of dithionite...
the signal of the oxidase is immediately abolished. Sulfide (0.005 M), thioglycolate (0.015 M), and other sulfhydryl reagents reduce more slowly. Borohydride decreases the intensity of the signal only to a minor extent (<20%). It may well be that the small portion of the copper reduced by borohydride represents again inactive material. It is of interest in this context that Yonetani (6) found 70 to 90% reduction by borohydride of the copper in his cytochrome oxidase preparations. This is contrary to the results obtained by chemical analysis by Griffiths and Wharton (8), whose findings agree with those that we obtained by EPR.

**Reaction with Inhibitors**—It was mentioned above that the copper of cytochrome oxidase is rapidly and completely reduced in the presence of azide or cyanide. We have not been able to observe reoxidation in the presence of these inhibitors even after prolonged aeration. Sands and Beinert (20), in their early studies of cytochrome oxidase by EPR, observed little effect with 0.003 M cyanide. As assumed by these authors, this level of cyanide is insufficient in concentrated solutions of the enzyme. Azide (0.02 M) has no effect on the EPR spectrum of cytochrome oxidase, whereas cyanide (0.01 M) produces a definite change (Fig. 2C). The signal is somewhat broadened, and the narrowly spaced fine structure toward lower field disappears. We would like to interpret this latter phenomenon as indicating reduction and formation of a Cu⁺ cyanide complex by that portion of the copper that is responsible for this fine structure and, we suspect, not in the same state as the copper of the native enzyme. It should be mentioned here that free Cu⁺ ions in solution are immediately reduced by cyanide with formation of Cu⁺ complexes; in other words, signals due to Cu⁺ complexes tend to disappear on addition of cyanide. The same is true for the Cu²⁺ hydroxylamine interaction. Cupric copper is readily reduced by this reagent. In contrast, as in the case with cyanide, the copper of cytochrome oxidase is not reduced by hydroxylamine, but is, however, strongly affected. Fig. 2E shows the drastic change that occurs on incubation with hydroxylamine. It should be pointed out that this signal was recorded at a lower amplification in order to fit it into the picture. The signal is sharpened, and the group of 9 lines toward lower field, which indicates interaction of the copper with 4 coordinated nitrogen atoms, becomes very prominent. The interpretation of this obviously complex spectrum remains ambiguous as long as more detail is not resolved. It is included only to show a strong interaction with hydroxylamine. It should be noted that hydroxylamine has been found to be a potent inhibitor of cytochrome oxidase (21).

**Addition of Cu²⁺ Ions**—Since the EPR spectrum of Cu²⁺ is sensitive to change of ligands, it appeared of interest to see what the spectrum of Cu²⁺ ions would be in the presence of cytochrome oxidase. Fig. 1, A and B showed spectra of copper complexes, which can exist in the medium in which cytochrome oxidase is dissolved. These spectra may be considered as blanks for the curve of Fig. 6B, which represents the spectrum of the oxidase after addition of a small amount of CuSO₄, approximately equivalent to the copper originally contained in the enzyme. Fig. 6A shows the corresponding spectrum of the untreated oxidase. It is clear from a comparison of Fig. 6A with 6B that the added Cu²⁺ ions produce a new signal at a slightly higher g value, i.e., at lower field, with considerable fine structure. This structure is reminiscent of the one seen in Fig. 2B in the purified oxidase and is probably due to interaction of the added copper with nitrogen atoms of the enzyme protein (15, 19). We can at least say that Cu²⁺ ions added to a solution of cytochrome oxidase find themselves in a constant environment, which is most probably provided by 4 nitrogen atoms. One could think here of a porphyrin structure (cf. (19)), but this appears very unlikely, as the porphyrin present in the molecule is definitely in the form of an iron porphyrin.

It may be anticipated here that treatment of cytochrome oxidase with alkali (Figs. 9 and 7B), acetone (Figs. 10B and 7D), or urea (Fig. 7C) produces changes in the EPR spectrum of cytochrome oxidase similar to those seen on addition of Cu²⁺ ions to the enzyme. The similarity of all of these spectra, illustrated in Fig. 7, suggests the following interpretation. Treatments that damage the oxidase, such as exposure to alkali, urea, or organic solvents, lead to a delocalization of a certain portion of the original copper of the native oxidase such as to destroy the copper-copper exchange interaction. This copper is then rebound at different sites at the protein in much the same way as are added Cu²⁺ ions where the copper ions are independent of one another. This copper then has properties different from those of the copper in the native enzyme. This can be shown by its behavior toward chelating agents.

**Interaction with Chelating Agents**—It is of interest that chelators for cupric or cuprous ions such as EDTA, 8-hydroxyquinoline, the cuproines, or diethylthiocarbamate have little or no effect on cytochrome oxidase activity (9). For EPR studies, we chose EDTA and bathocuproinedisulfonate as strong chelators for cupric and cuprous ions, respectively. BCS is of particular interest, since Yonetani (6) has shown that it binds and fixes 60 to 70% of the copper in his enzyme preparations in the cuprous state. According to Yonetani, this accounts for all of the copper.
that is reducible by substrate. BCS was obtained from the G. Frederick Smith Chemical Company, Columbus, Ohio.

Fig. 6C shows the EPR spectrum of the cytochrome oxidase of Fig. 6A after addition of BCS. There is relatively little change. Fig. 6D shows the spectrum of oxidase plus added Cu^{2+} ions (of Fig. 6B) after addition of BCS. Again, there is little change, except for some fading of the fine structure toward lower field, which is due to extraneous copper. When cytochrome c and ascorbate are added to this mixture of enzyme, CuSO_{4}, and BCS, the spectrum of Fig. 6E results. The copper of the oxidase as well as the added copper are reduced. The most significant spectrum in this series is that of Fig. 6F. After aeration and exhaustion of ascorbate, the signal characteristic of the native copper of the oxidase reappears, whereas the secondary peak and fine structure of the added Cu^{2+} ions are lost. We conclude from this experiment, which was repeated several times, that the native copper of the oxidase in its cuprous form does not react with BCS, whereas the copper bound to the oxidase in the linkage that produces an extra peak and the narrowly spaced fine structure at low field is bound and fixed in the cuprous state by BCS. Fig. 9 will show that this is also true for the alkali-treated enzyme. Again, our finding that BCS does not react with the copper of cytochrome oxidase is in agreement with the spectrophotometric tests of Griffiths and Wharton (8), who could not detect any appreciable complex formation of this copper with BCS, contrary to Yonetani's observations with his enzyme preparation.

In Fig. 8, EPR spectra are reproduced that were obtained on addition of EDTA and Cu^{2+} ions to the enzyme. Fig. 8, B and A, shows the spectra of the untreated enzyme with and without the addition of EDTA, respectively. There is no significant change, which is consistent with the fact that EDTA does not inhibit cytochrome oxidase. On addition of a small quantity of CuSO_{4} to the solution, which now contains EDTA and cytochrome oxidase, we observe the spectrum of oxidase copper plus that of the EDTA-Cu^{2+} complex (Fig. 8C). The same result is obtained when CuSO_{4} is added to the enzyme first and EDTA thereafter. For comparison, the spectrum that appears when Cu^{2+} ions are added to the oxidase in the absence of EDTA is shown in Fig. 6B. The spectrum of the EDTA-Cu^{2+} complex is seen in Fig. 8D after reduction of the oxidase copper by substrate. Contrary to the situation in the absence of EDTA (Fig. 6E), extraneously added Cu^{2+} is not reduced in the presence of the oxidase, cytochrome c, and ascorbate when EDTA is present. It is known that EDTA prevents the reduction of Cu^{2+} by ascorbate or, in other words, that EDTA prevents the autoxidation of ascorbate in the presence of Cu^{2+}. Fig. 8E then shows the spectrum obtained after exhaustion of the added ascorbate and reoxidation of cytochrome oxidase. The spectrum of Fig. 8C is restored.

The two experiments of Figs. 6 and 8 clearly illustrate the difference in reactivity of at least two types of copper that may be associated with the oxidase: (a) the copper of the native enzyme in its original binding state and (b) secondarily bound extraneous copper ions. In the presence of BCS, both species of copper are reduced by substrate, but only the copper of the native enzyme can be reoxidized; in the presence of EDTA, only the copper of the native oxidase can be reduced by substrate and then reoxidized, whereas the other species is fixed in the cupric state.

It was now of great interest to see how the copper of the native enzyme would behave toward these chelators after it had been modified in its binding state by damaging treatment. Accord-
is typical of the original copper of the oxidase. The signal of almost complete reduction. Aeration then reoxidizes part of the secondarily bound copper. Little change accompanies addition of the pH to a value of approximately 12, and Fig. 9C, the ing to EPR spectra (cf Figs. 6B, 7, 9C, and 10B), this copper is present. The strong peak toward lower field and the smaller associated fine structure are eliminated, Fig. 9G shows the same spectrum as Fig. 9F, but at higher amplification (× 2); H, sample treated with alkali and reneutralized, similar to C above, 1 hour after addition of 0.6 µmole of EDTA, pH 7.4.

ing to EPR spectra (cf Figs. 6B, 7, 9C, and 10B), this copper is now in a binding state similar to that of extraneously added copper. An experiment designed to answer this question is illustrated in Fig. 9. Fig. 9A shows the spectrum of the untreated enzyme, Fig. 9B, the spectrum of the same enzyme after adjustment of the pH to a value of approximately 12, and Fig. 9C, the spectrum after immediate neutralization to pH 7.8. At this point there appear toward low field the fine structure and a second minor peak, which we consider typical for delocalized and secondary bound copper. Little change accompanies addition of BCS (Fig. 9D). Fig. 9E shows the spectrum at the state of almost complete reduction. Aeration then reoxidizes part of the copper (Fig. 9F). It is significant that the signal that reappears is typical of the original copper of the oxidase. The signal of what we called “secondarily bound” copper is abolished. This copper appears to be fixed in the cuprous state by BCS (Fig. 9D). Fig. 9E shows the spectrum at the state of Fig. 9C, namely, to the alkali-treated and reneutralized, similar to C above, 1 hour after adjustment of 0.6 µmole of EDTA, pH 7.4.

To make it more evident that the peak at lower field and the associated fine structure are eliminated, Fig. 9G shows the same spectrum as Fig. 9F but at higher amplification. Fig. 9H finally shows the effect of EDTA when this chelator is added at the state of Fig. 9C, namely, to the alkali-treated and reneutralized enzyme. It will be recalled that Fig. 8B showed no effect of EDTA on the spectrum of the untreated oxidase. Fig. 9H, however, makes it clear that after damaging treatment, resulting in an apparent delocalization and secondary rebinding of copper, EDTA is able to form a complex with a sizable portion of the copper present. The strong peak toward lower field and the smaller lines farther to the right represent the EDTA-Cu⁺⁺ complex (cf. Fig. 8, C and E). An analogous effect of EDTA was seen after urea treatment. In the series of Fig. 9, it is particularly interesting that only a relatively small portion of the original copper of the oxidase is reoxidizable in the presence of BCS. This shows that the copper is sensitive to certain treatments that convert it to a considerable extent into a form that will react and can be fixed in the cuprous state by BCS.

Treatment with Acetone—It is known that the heme components of most cytochromes, including cytochrome a, can be extracted with acidified acetone. It was therefore of interest to learn whether the copper associated with cytochrome oxidase is extracted by this agent. Fig. 10 illustrates experiments designed to answer this question. Fig. 10A shows the spectrum of the untreated enzyme, Fig. 10B, the spectrum of the residue after treatment with acetone alone, and Fig. 10C, that of the concentrated extract after repeated treatment of the first residue with acid acetone. This treatment extracts the heme. It is apparent that the copper is not extracted from the protein by this procedure. When, however, acidified acetone is used immediately, the copper is extracted together with the heme (Fig. 10D). The residue shows no signal (not shown).

The spectrum of Fig. 10B is of interest with respect to the foregoing discussion on the change of the EPR spectrum of the copper in cytochrome oxidase after damaging treatment. We recognize that the characteristic change, which we observed after alkali treatment and after addition of extraneous copper to the enzyme, also results from acetone treatment of the enzyme. This further supports our contention that this type of spectrum is no longer typical of the native enzyme and that the copper producing the new features in the spectrum is in a state different.
from that of the original copper and should therefore be expected
to react differently.

**DISCUSSION**

The EPR spectrum of cytochrome oxidase is similar to that of
other known copper proteins, such as laccase (22) or ceruloplas-
min (15), in which copper is present in the cupric form. The g
value of the oxidase \( g_{\text{ox}} = 2.03 \) is among the lowest observed
for copper proteins and complexes (15). As was more extensively
discussed above, the lack of nuclear hyperfine structure in the
spectrum of the enzyme indicates that at least 2 copper ions are
in close proximity in the enzyme and undergo an exchange inter-
action (13, 14). We consider it possible that these vicinal ions
are in opposite oxidation states, viz. 1 cupric and 1 cuprous.
Support for these conclusions comes from the results of integrations
and quantitative evaluations of the spectra, which leave approxi-
imately two-thirds of the chemically determined copper unac-
counted for. The evidence that accrues from these EPR studies,
namely, that there must be at least 2 copper atoms in close prox-
imity in every molecule of cytochrome oxidase, appears to us of
considerable interest with respect to the interaction of the en-
zyme with oxygen, in particular since conventional methods of
molecular weight determination on the native and active enzyme
fail and thus cannot lead to an estimate of the moles of copper
present per mole of enzyme. If copper is indeed the component
of the oxidase that interacts directly with oxygen, it is difficult
to visualize an interaction of a single copper atom with the oxy-
gen molecule.

The fact that the spectrum of the isolated oxidase is almost
identical with that shown by mitochondrial fragments (23),
which contain a functioning cytochrome chain and are capable
of oxidative phosphorylation, supports the view that the environ-
ment of the copper has not been significantly changed during
purification. We would like to emphasize particularly that the
narrowly spaced fine structure (apparently 9 lines due to coordi-
nation with nitrogen) toward the low field side of the signal is
not observed in these spectra of particles and highly purified ac-
tive oxidase. Pronounced fine structure in this region is, how-
ever, seen when the oxidase has been exposed to alkali, acetone,
or urea or when traces of \( \text{Cu}^{2+} \) ions have been added to the en-
zyme. For comparison, the corresponding spectra are shown in
Fig. 7. As will be discussed below, the copper responsible for
the fine structure of the EPR signals of Fig. 7, B, and C, reacts
in a way different from the original copper of the oxidase. This
is in fact not surprising, as the copper finds itself in a different
environment in either situation, and the interaction between vic-
inal copper atoms is only characteristic of the native state.

The EPR spectra leave no doubt that the copper in cytochrome
oxidase is reduced to the cuprous form when the enzyme is re-
duced by substrate. This has been confirmed by chemical analy-
alsis (9). Furthermore, the experiments in which reduction of the
heme component of the oxidase was followed by optical spectro-
copy simultaneously with EPR measurements on equally treated
aliquots show that the oxidation state of the copper closely fol-
loows that of the heme: from complete oxidation through the
steady state, to almost complete reduction, and back to the ox-
idized form. Reduction of both copper and heme shows de-
pendence on cytochrome c concentration. It should be under-
stood, however, that the time resolution in these experiments is
far from sufficient to compel the conclusion that the copper of
the oxidase is an obligatory intermediate on the path of electrons
to oxygen.

The EPR spectra also show that known inhibitors of cyto-
chrome oxidase, such as cyanide and hydroxylamine, produce
definite changes in the binding state of the copper. No effect
was observed with azide. However, after addition of cyanide or
azide, reduction of the copper by substrate proceeded consider-
ably faster, and no reoxidation could be observed on prolonged
aeration. This behavior again parallels that of the heme com-
ponent of the oxidase.

It was previously shown that neither BCS nor EDTA, which
are known as strong chelators of cuprous and cupric copper, re-
spectively, inhibit the activity of cytochrome oxidase (9). Again
in line with this are the observations that the EPR spectrum of
cytochrome oxidase is unaffected by the presence of these re-
agents and that the copper of the enzyme can pass through a
complete cycle of reduction by substrate and reoxidation by oxy-
gen without any irreversible change in its EPR spectrum or, in
other words, in its state. Copper, which is added to the oxidase,
however, and which is also bound to the enzyme according to its
EPR spectrum, reacts differently. In the presence of EDTA it is
not reduced by substrate, and in the presence of BCS it is re-
duced and fixed in the reduced state as the BCS complex while
the activity of the enzyme is unimpaired. Yonetani (6) con-
cluded from experiments with his oxidase preparation that this
behavior was characteristic of the major portion of the copper in
the enzyme and that probably most, if not all, of the copper
found was an impurity, unrelated to the function of the enzyme.
Yonetani considered irrelevant the fact that the copper associ-
ated with the oxidase was capable of undergoing oxidation-reduc-
tion that is in some way coupled to that of the heme.

It may be well to emphasize again at this point that with our
cytochrome oxidase preparations and under the conditions of
resolution of our experiments, we have never observed disparity
in the oxidation state of copper and heme. Only the reagents
that reduce the heme are also able to reduce the copper. It may
be recalled that borohydride, for instance, is incapable of reduc-
ing the heme and also fails to reduce the copper of the enzyme.

We have also confirmed the previous experiments of Sands and
Beinert (20), which showed that cytochrome c (reduced with
palладium and hydrogen) does indeed reduce both copper and
heme in our cytochrome oxidase preparations, contrary to the
findings of Ehrenberg and Yonetani (10) with their preparation.

The observation cited above, namely, that certain treatments
of the oxidase, of which exposure to high pH is an example, may
modify part of the copper in its binding state and may then
make it susceptible to chelation by BCS in the cuprous form
might provide an explanation for the findings of Yonetani. In
these cases, the copper would then behave like copper ions that
are added to the enzyme and are bound in a state different from
that of the copper in the native enzyme, namely, in the state
which produces the fine structure at low field (Fig. 7). Our sug-
gested explanation receives support from the recently published
EPR spectra (10) of Yonetani’s enzyme preparation, which re-
semble the spectra that we consider characteristic of damaged
enzyme. It is stated by Ehrenberg and Yonetani, concerning
these spectra: “The hyperfine spacing...corresponds to a split-
ting constant of 0.019 cm⁻¹, which is of the magnitude ordinarily

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8 The molecular weight of the native enzyme is not known.
9 The preparations used in these studies (cf. (5)) contained approxi-
amately 8 mmoles of heme and 8 mpatoms of copper per mg of
protein.
found for cupric complexes. 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. They suggested that there is a relation between the enzymatic activity and the high degree of delocalization of the unpaired hole as evidenced by the small splitting constant. The normal splitting constant of the ESR [electron spin resonance] 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. We would like to add here that, according to our findings and the spectra published by these authors, they measured the hyperfine splitting constant of that copper in their enzyme that is no longer in the native state and thus come rightly to the conclusions just quoted.

We conclude from our experiments that the idea of copper being a component oxidation-reduction catalyst of cytochrome oxidase should not be prematurely discarded on the basis of rather limited evidence. The experiments described in this paper certainly support the view that copper has the qualities of a functional constituent of the oxidase: (a) the typical Cu²⁺ signal found by EPR in mitochondria and submitochondrial particles is concentrated during the isolation of cytochrome oxidases; (b) Cu²⁺ in all of these preparations undergoes oxidation-reduction parallel to that of the heme components, and, under certain conditions, the reaction rates are very similar; (c) known inhibitors of cytochrome oxidase interfere with the oxidation-reduction of the copper in the manner expected from studies of over-all activity of the enzyme, and in some cases direct interaction with the copper can be demonstrated by changes in the EPR signal. It might be mentioned in closing that it is the rule rather than the exception that oxidases of the type of cytochrome oxidase are copper proteins (24), and the conclusion drawn from our studies, that at least 2 copper atoms are present in the enzyme in close proximity, make the idea of a concerted interaction of these copper atoms with 1 oxygen molecule rather attractive.

Because of the great interest that such a copper complex in cytochrome oxidase deserves, we are attempting to determine the number of interacting copper atoms, their ionic states, and the nature of their interaction more precisely.

**SUMMARY**

The interaction of the copper associated with cytochrome oxidase with cytochrome c and ascorbate, chemical reducing agents, oxygen, and certain inhibitors and chelating agents has been studied by electron paramagnetic resonance (EPR) spectroscopy. At least part of the copper present in the enzyme is in the cupric form, and the spectra are interpreted as indicating that 2 or more copper atoms are in close proximity in the enzyme. The copper is readily reduced to the cuprous form by substrate or chemical reducing agents. In these reactions, the oxidation state of the copper parallels that of the heme component of the enzyme. It is concluded from a comparison of paramagnetic resonance spectra, after damaging treatment of the enzyme or after addition of Cu⁺ ions to the enzyme, that the copper of the native enzyme is delocalized by certain treatments and rebound in a fashion similar to extraneously added Cu⁺ ions. This secondarily bound copper, although still reducible by substrate, has properties different from that of the copper in its original binding state in the native enzyme. The relative proportions of these two species of copper in a cytochrome oxidase preparation will determine the apparent reactivity of the copper in cytochrome oxidase towards a variety of reagents and particularly with regard to the interaction of the reduced species with oxygen.

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