Electron Spin Resonance of
Some Bacterial Respiratory Membranes

Trevor Lund and J. Barrie Raynor

Department of Chemistry, The University, Leicester, England

Abstract

Electron spin resonance measurements have been carried out on extracts from eight different bacterial respiratory membranes. The available evidence suggests that the copper is probably associated with the terminal cytochromes. From the Fe^{3+} ($g = 4.3$) signal, copper: cytochrome ratios and the absence of any detectable copper in the ESR spectra at $77°$ K under varying oxidation and reduction conditions, it is concluded that most of the copper in the membranes is paired with other coppers or more probably with a high-spin ferric ion with the copper–metal distance not greater than 5 Å.

Cytochrome oxidase from animal sources has been extensively studied and discussed [1–7]. Copper has been implicated in its function but it has been found difficult to interpret the ESR properties of the copper involved. Only half of the copper present and necessary for the activity of the enzyme is detected by ESR and the copper that is detected has unusual g- and A-values. Yong and King have suggested [6] that the copper not detected by ESR is close to the heme a_3 and participating in a heme–copper interaction such that both its undetectability by ESR and the anomalous ESR behavior of a_3 is explained. The copper observed by ESR is thought to be sandwiched between a and a_3 in the electron-transport chain, and participating in a heme copper–heme interaction.

Recently, Hartzell et al. [7] have suggested that heme a_3 is undetectable by ESR because it is involved in an antiferromagnetic interaction with the ESR-undetectable copper, and the signal previously

assigned to it belongs in fact to heme a. No suggestions are made, however, for the relative strengths of the other possible interactions between the components of the cytochrome. Malström [1], on the other hand, draws the distinction between long-range and short-range forces, suggesting that the heme a_3 and the ESR-undetectable copper are physically close to each other and participating in short-range magnetic spin-spin interactions. This explains the undetectability by ESR of the first two components and the unusual spectrum of the third. Heme a is then thought to be physically remote from this cluster (>10 Å), interacting only *via* long-range allosteric effects which simultaneously explain its kinetic behavior and its sharp ESR spectrum—an idea not inconsistent with that of Yong and King.

Little work has been done on bacterial extracts, where, since the combination of cytochromes in the respiratory membrane does not necessarily contain aa_3, the configuration of the heme and copper groups will not necessarily be the same as described for bovine and pigeon-heart muscle preparations. It is known from atomic absorption analysis [8] that even if the respiratory membrane does not contain the aa_3 unit, copper is still present in approximate stoichiometry with the terminal cytochromes. The question of the role of copper in the respiratory membranes of bacteria then requires further investigation. Because of the difficulty of isolation of pure cytochromes, the measurements reported in this paper were made on respiratory particles which have the advantage of containing the respiratory membrane in a form probably little modofied from that *in vivo*. As copper was the chief object of interest, the ESR observations were made at $77°$ K where the copper was expected to be easily detectable [2, 3, 6] and where the signals from iron were expected to be weaker and to interfere less. The bacteria chosen for this study were selected to cover a range of combinations of terminal cytochromes.

Results

The ESR spectra of each preparation in both the oxidized and reduced states had features in common. Absorptions attributable to high-spin ferric ions at $g = 4.3$ were often detected in the oxidized samples. Upon reduction with either malate, succinate, or NADH, an additional sharp line at $g = 2$ due to the semiquinone radical was always present. In none of the bacterial preparations was any sign of a Cu^{2+} signal seen either in the oxidized or reduced forms.

A small sample (~6 mg) of purified freeze-dried beef-heart cytochrome oxidase was used to check on the sensitivity of the ESR spectrometer. A copper spectrum was obtained identical with that reported by Beinert [3]. The copper spectrum was sufficiently strong that

we would feel confident of detecting a similar signal less than one fifth of the size. The amount of copper responsible for this signal was determined by atomic absorption analysis.

The results of atomic absorption analysis for copper are shown in Table I. The amount of copper present in each sample in the central region of the ESR cavity has been estimated from the copper concentration measured. It can be seen that each of the samples of bacterial particles contains a comparable amount of copper to the cytochrome-oxidase sample used as a reference.

TABLE I. The concentration of copper in the samples examined, as measured by atomic-absorption spectrophotometry, and the amount of copper in the ESR sample actually used

Sample	Terminal cytochromes			Copper con-centration (μg/ml)	μg Cu in sample
A. vinelandii	a_1	a_2	o	6.3	0.95
E. coli		o		40	6.0
Ps. ovalis		o		3.5	0.53
A. Lwoffi		o		4	0.6
B. licheniformis	aa_3	o		22	3.3
B. megaterium	aa_3	o		5	0.75
M. thermosphactum	aa_3			20	3.0
M. thermosphactum[a]	aa_3			0.027 μg/mg	1.6
B. subtilis	aa_3			_[b]	
Cytochrome oxidase[a]	aa_3			0.25 μg/mg	1.5

[a] Freeze-dried sample.
[b] Not measured in this work, but the Cu/a_3 ratio has been given as 1.6 [8].

Discussion

In none of the bacterial particles examined have we found any sign of a copper ESR spectrum, although we have checked the sensitivity of our spectrometer and know that we should be able to detect (if the copper is Cu^{2+}) about a quarter of the copper found by atomic absorption spectroscopy in our weakest sample (*Pseudomonas ovalis*) and about one-fortieth of the copper found in the strongest sample (*Escherichia coli*). We are forced to conclude that only a small proportion of the copper present is in a form potentially detectable by ESR (Cu^{2+}), e.g. less than 2.5% of the copper in *E. coli* and less than 25% in *Ps. ovalis*. Therefore, if the copper present is associated with respiration in these bacteria then it is in a different environment from that found in

cytochrome oxidase from animal sources. In order to be absolutely certain of the role of copper in bacterial respiratory membranes it would be necessary to isolate smaller fragments of the respiratory chain to ascertain whether the copper is in fact associated with the terminal cytochromes. Nevertheless, there is some evidence that the copper is associated with the terminal cytochrome, for example, the copper signals observed in submitochondrial particles [9], mitochondria, and whole-cell samples [10] from beef, pigeon, and rat heart have been assigned to cytochrome oxidase, although in intact algae they have been assigned to plastocyanin [11].

The evidence that most of the copper is associated with ther terminal cytochrome, at least in some of the bacterial enzymes, is as follows. From total copper and terminal cytochrome analysis, Meyer [8] has deduced that the total copper to terminal cytochrome ratios vary up to about 3.1:1. For example, *Microbacterium thermosphactum* and *Bacillus subtilis* have ratios 3.1:1 and 1.6:1. Since both have cytochrome $a\, a_3$ as terminal cytochrome, and since the ratios are not dissimilar to those found in mammalian enzymes with the same terminal cytochrome (ratio cytochrome copper:cytochrome::2:1), then it could be that the bacterial cytochrome aa_3 also has two copper atoms in it. In *M. thermosphactum*, the total copper:cytochrome ratio of 3.1:1 might indicate that two copper atoms are in the terminal cytochrome and one in the noncytochrome oxidase region of the respiratory system. Nevertheless, all copper atoms seem to be ESR nondetectable which suggests pairing with each other or with another metal. Similar tentative conclusions may be drawn from other bacterial enzymes with terminal cytochrome o (e.g., *Acinetobacter lwoffi*) which have total copper:cytochrome ratios very close to 1 suggesting that the sole copper atom is associated with the terminal cytochrome.

In contrast to the mammalian enzymes where there is ESR detectable copper, our results for those bacterial enzymes which we have studied show that if they do contain copper, either in the terminal cytochrome or in the nonrespiratory area of the enzyme, then as far as the oxidized form is concerned, most of it must be involved in short range $Cu^{2+}-Cu^{2+}$ or $Cu^{2+}-Fe^{3+}$ spin–spin interactions sufficiently strong as to imply an interionic distance of $\leqslant 5$ Å [12, 13]. Support for this comes from the surprising observation that the $g = 4.3$ high–spin ferric signal often increases in intensity upon reduction of the bacterial particles. This was particularly noticeable with those enzymes having terminal cytochrome. This could come about if there was selective reduction in a strongly coupled $Cu^{2+}-Fe^{3+}$ pair such that the cupric ion was reduced by the malate or succinate but the ferric ion was not, so allowing a signal to be observed from the ferric ion. Such a reduction might be accompanied by a rearrangement of the ligands so as to separate the two metal centers and allow the Cu^{+} and the Fe^{3+} to be separately stabilized [14]. Griffith

[12] has explained how one of the $Cu^{2+}-Fe^{3+}$ heme pairs in cytochrome oxidase is probably antiferromagnetically coupled in the oxidized state, while in the partially reduced state, the Cu^{2+} ion is reduced but not the Fe^{3+} ion, the only ESR signal observed being that due to the freed high-spin ferric heme. In our enzymes the Fe^{3+} ion must be in a highly stabilized, well shielded, and unique ligand environment in a side chain not susceptible to reduction and containing ligands such as carboxylic acids, oxines, or amino acids [15]. Redox potentials applicable for such a reaction suggest that it is feasible [14], there being ample evidence for $Cu^{2+}-Fe^{3+}$ pairs with, probably, hydroxyl bridges [16].

Alternatively, the increase in Fe^{3+} signal strength could arise from a change in relaxation time caused by a change in the ferric ligand coordination or stereochemistry. This model would necessitate $Cu^{2+}-Cu^{2+}$ or $Cu^{2+}-Fe^{3+}$ heme antiferromagnetically coupled pairs. If reduction took place at the copper sites and was accompanied by an alteration in the ligand environment of the nonheme ferric without reduction, then this could account for the increase in the ferric signal upon reduction. Malmström [1] has tentatively suggested that the signal assigned to copper in mammalian cytochrome oxidase may not be due to copper at all but originates from a low-spin Fe^{3+}. In this case the copper ions, being undetectable, might be comparable to the nonparamagnetic pair found in the blue oxidases [17]. A mixed-valence sulfur-bridged pair of the type suggested by Hemmerich [18] and others does not seem likely—a reasonably sharp ESR spectrum would be expected. In the reduced form all the copper must be as Cu^+. We must conclude that at least in the high-copper samples, the copper environment is significantly different from that found in mammalian cytochrome oxidases. Nevertheless, the experiments reported in this paper strongly suggest that Cu–metal interactions exist but raise more questions than are answered. The system clearly has considerable complexity.

Acknowledgments

References

1. B.G. Malmström, *Quart. Rev. Biophys.*, 6 (1974) 389.
2. T. Vänngård in *Biological Applications of Electron Spin Resonance*, H.M. Swartz, J.R. Bolton and D.C. Borg, eds., p. 411, Wiley-Interscience, New York (1972).
3. H. Beinert in *The Biochemistry of Copper*, J. Peisach, P. Aisen and W.E. Blumberg, eds., p. 213, Academic Press, New York (1966).

4. H. Beinert, C.R. Hartzell and W.H. Orme-Johnson, in *Probes of the Structure and Function of Macromolecules and Membranes,* B. Chance, T. Yonetani and A.S. Mildvan, eds., p. 575, Academic Press, New York (1971).

5. B.F. Van Gelder, W.H. Orme-Johnson, R.E. Hansen and H. Beinert, *Proc. Natl. Acad. Sci., U.S.A.,* **58** (1967) 1073.

6. F.C. Yong and T.E. King, *J. Biol. Chem.,* **247** (1972) 6384.

7. C.R. Hartzell, R.E. Hansen and H. Beinert, *Proc. Natl. Acad. Sci.,U.S.A.,* **70** (1973) 2477.

8. D.J. Meyer, Ph.D. Thesis, University of Leicester, England (1972).

9. N.R. Orme-Johnson, W.H. Orme-Johnson, R.E. Hansen, H. Beinert and Y. Halefi, *Biochem. Biophys. Res. Commun.,* **44** (1971) 446.

10. N.R. Orme-Johnson, R.E. Hansen and H. Beinert, *J. Biol. Chem.,* **249** (1974) 1922.

11. J.W.M. Visser, J. Anesz and B.F. Van Gelder, *Biochim Biophys. Acta,* **333** (1974) 279.

12. J.S. Griffith, *Mol. Phys.,* **21** (1971) 141.

13. A.P. Ginsberg, *Inorganica Chim. Acta Reviews,* **5** (1971) 45.

14. L.E. Bennett in *Prog. in Inorg. Chem.,* **18**, S.J. Lippard, ed., p. 1, John Wiley and Sons, New York (1973).

15. R.J.P. Williams, *Inorganic Chemistry,* **2**, p. 313, Oxford University Press, London (1966).

16. R.J.P. Williams in *The Biochemistry of Copper,* J. Peisach, P. Aisen and W.E. Blumberg, eds., p. 131, Academic Press, New York (1966).

17. R. Malkin and B.G. Malmström, *Adv. Enzymol.,* **33** (1970) 177.

18. C. Sigwart, P. Hemmerich and J.T. Spence, *Inorg. Chem.,* **7** (1968) 2545.