ELECTRON PARAMAGNETIC RESONANCE STUDIES OF IRON REDUCTION AND SEMIQUINONE FORMATION IN METALLOFLAVOPROTEINS*

K. V. Rajagopalan, Victor Aleman and Philip Handler
Department of Biochemistry, Duke University, Durham, N. C.,
W. Heinen, G. Palmer and Helmut Beinert
Institute for Enzyme Research, University of Wisconsin, Madison, Wis.

Received May 28, 1962

A component of mitochondria and of isolated flavoproteins of the respiratory chain which becomes apparent on reduction by appropriate substrates and which is recognizable by a characteristic asymmetric EPR signal at $g_{\parallel} = 2.00$, $g_{\perp} = 1.94$, was described by Sands and Beinert (1960 a, b). The properties of this component and its EPR signal, described by Beinert and Lee (1961) and by Beinert (1962), suggest that it is a paramagnetic ion. Since all of the materials in which the signal was detected contain significant amounts of iron, and in some instances no heavy metal but iron, it was postulated that a reduced form of iron is responsible for the EPR signal. A signal of similar characteristics which appears upon reduction by xanthine of milk xanthine oxidase, an iron- and molybdenum-containing flavoprotein, has been observed by Bray et al. (1961) and by Beinert[1]. These observations have suggested that, in a variety of flavoproteins, there is a similar, specific form of iron complex of unknown structure, which is alternately reduced and reoxidized during the normal catalytic activity of these systems.

Concomitant with these developments, a group of metal-containing flavoproteins, specifically milk xanthine oxidase (Fridovich and Handler, 1958 a, b, 1961, 1962), rabbit liver aldehyde oxidase (Rajagopalan, Fridovich and Handler, 1962; Rajagopalan and Handler, 1962) and dihydroorotic dehydrogen-

*Supported by research grants from the National Institutes of Health (USPHS) (RG-91, RG-5073, RG-6762, A-2512) and by AEC contract AT(11-1)-909.

[1] unpublished

ase (Friedman and Vennesland, 1961; Aleman and Handler, 1962) was under investigation. While differing in many facets of their specificity and structure, these three flavoproteins exhibit, in common, a group of related properties, each of which appears to be a consequence of the reduction of molecular oxygen to the superoxide anion ($O_2^{\cdot-}$). In the presence of its substrates, each of these enzymes initiates the autoxidation of sulfite. This process is dependent upon the formation of the superoxide anion or its protonated form, the perhydroxyl radical ($HO_2^{\cdot}$) (Fridovich and Handler, 1958 a) and cannot be initiated by simple formation of flavin semiquinones (Fridovich and Handler, 1961). All three enzymes exhibit an absolute dependence upon the presence of molecular oxygen in order to accomplish the reduction of cytochrome c, which appears to be effected by the same oxygen radicals. Each of these three enzymes initiates the process of chemiluminescence when enzyme, substrate and oxygen are incubated in the presence of dimethylbiacridylium nitrate. Considerable evidence indicates that this process, also, reflects formation of oxygen radicals as a consequence of enzyme activity (Greenlee, Fridovich and Handler, 1962).

Since the specific EPR signal of Sands and Beinert was found upon reduction of one of the enzymes of this group by its substrates, viz., xanthine oxidase, it was of interest to examine rabbit liver aldehyde oxidase and dihydroorotic dehydrogenase by EPR spectroscopy in similar fashion.

Low temperature EPR spectroscopy was performed essentially as described by Beinert et al. (1962) and by Beinert and Sands (1961). Free radical concentration was estimated by double integration of the derivative signal and comparison to a nitrosyl disulfonate standard. In the experiments with dihydroorotic dehydrogenase, samples were frozen immediately after addition of DPNH and then examined, consecutively, by EPR (at -175°) and optical reflectance spectroscopy (at -90°) before any changes in the oxidation-reduction state of the enzyme could occur. A Cary model 14 spectrophotometer, equipped with a modified diffuse reflectance attachment, was employed in the

latter studies. Spectra of liquid samples were obtained with a Cary model 14 spectrophotometer.

Figure 1 shows that reduction of both aldehyde oxidase and dihydroorotic dehydrogenase by their respective substrates resulted in the appearance of the characteristic asymmetric signal at $g_{\perp} = 1.94$. In addition, with each of these enzymes, organic free radicals (g=2.00) appeared, as had previously been described for the case of xanthine oxidase (Bray et al., 1961). Reduction of aldehyde oxidase also yielded a signal characteristic of a reduced form of molybdenum (g=1.97). The signals at g=1.94 have the same general properties previously observed by Beinert and Sands (1960) and by Beinert and Lee, (1961), i.e., the specific g value and the pronounced sensitivity to temperature, although there are minor variations in signal shape.

The fact that this characteristic type of signal has been found in an increasing number of different iron-containing flavoproteins, but not with flavoproteins which lack iron, strengthens the contention that iron is the paramagnetic species responsible for its appearance and suggests strongly that a rather specific type of catalytically active iron complex occurs in all of these enzymes. Of the group, dihydroorotic dehydrogenase appears to be the member of simplest structure in that it is not known to contain any metal other than iron and contains only one iron atom per molecule of flavin (two moles of iron and flavin per mole of enzyme). Hence, the appearance of the characteristic signal upon reduction of this enzyme offers perhaps the most convincing single argument for the identification of the EPR signal at $g_{\perp} = 1.94$ as that of an iron complex.

It is noteworthy that, in each instance in which the specific signal has thus far been observed, the enzyme contains at least two atoms of iron per molecule. This correspondence leads to the suggestion, which has also been made on other grounds (Rajagopalan and Handler, 1962), that the active complex, may involve two vicinal iron atoms. This concept is in keeping with the observation that the signal is irreversibly lost when enzymes of the group considered here are treated with 6 N urea, or after proteolysis (Beinert

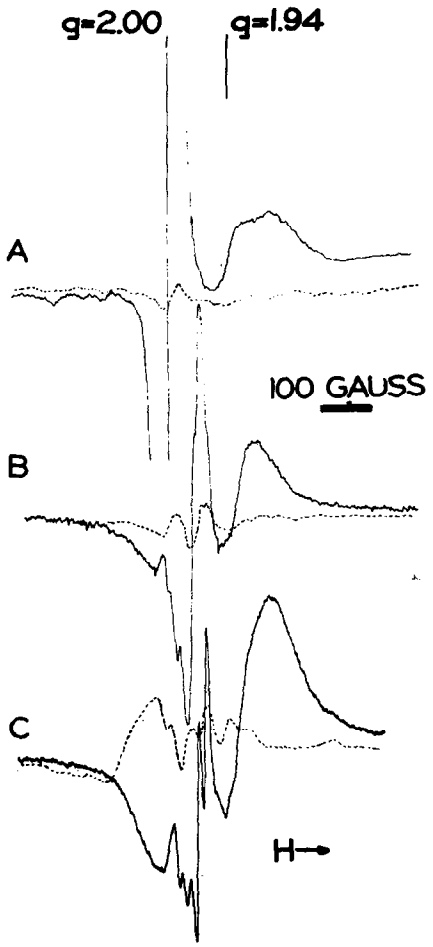

Figure 1 EPR spectra (first derivative, -175°) of metalloflavoproteins. Broken lines: untreated; solid lines: 5 minutes after additions at 0°. A, dihydroorotic dehydrogenase (33 mμmoles of bound flavin) in 0.35 ml of 0.2 M phosphate of pH 6.5; addition: 1.7 μmole of DPNH; B, aldehyde oxidase (84 mμmoles of bound flavin) in 0.20 ml of 0.05 M phosphate of pH 7.8; addition: 0.7 μmole of acetaldehyde. The untreated control shows a small Mo^{5+} signal. This signal increases after addition of substrate and covers up a small free radical signal at g=2. C, xanthine oxidase, 10 mg in 0.15 ml of 0.1 M phosphate of pH 7.5; addition: dithionite. The untreated control shows the presence of some contaminating Cu^{2+} ($g \sim 2$) and some Mo^{5+} (g=1.97). This signal becomes more prominent on reduction.

and Lee 1961). At the same time, it is in keeping with the mechanism proposed by Rajagopalan and Handler (1962) to account for reduction of oxygen both to peroxide and to the superoxide anion by certain enzymes of this group.

It is apparent from Figure 1 that significant formation of an organic free radical occurred upon reduction of dihydroorotic dehydrogenase by its

substrate. Friedman and Vennesland (1961) had previously observed an increased absorption between 500 and 600 mμ upon addition of substrate to this enzyme. Similar spectral changes had been reported by Beinert (1957) and Beinert and Sands (1961) for flavins and for certain flavoproteins. These were ascribed to semiquinone formation in some instances. To confirm this concept, optical reflectance and EPR spectra were recorded at the same state of reduction. The results are summarized in Figures 2 and 3.

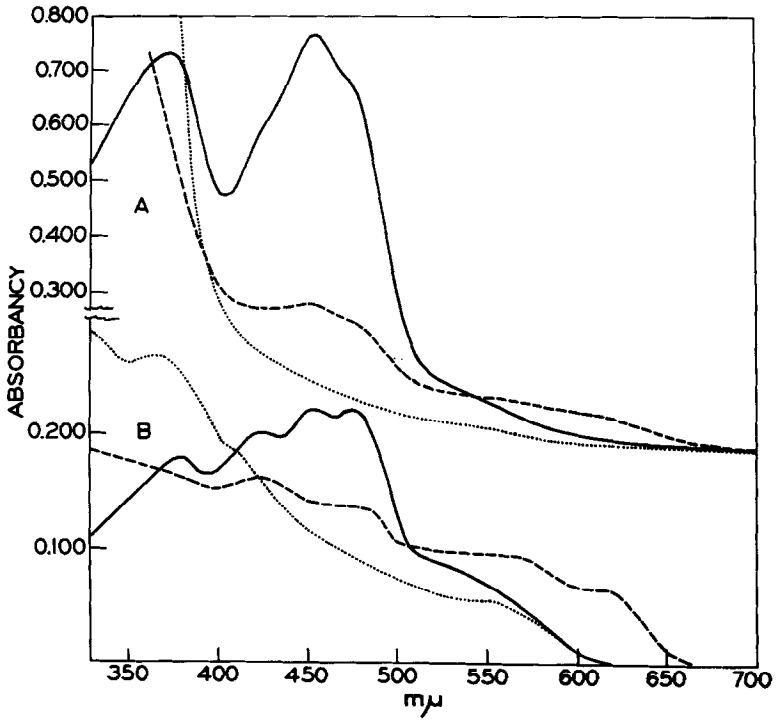

Figure 2 Absorption spectra of dihydroorotic dehydrogenase recorded on liquid sample by absorption spectroscopy (A) and on frozen sample with diffuse reflectance method (B). For (A) protein corresponding to 43 mμ moles of bound flavin was used in 1 ml of 0.2 M phosphate of pH 6.5 (aerobic, light path 1 cm); ——— untreated; --- after addition of 0.43 μmole of DPNH; ····· after addition of dithionite to the previous sample. For (B) protein corresponding to 33 mμ moles of bound flavin was used in 0.35 ml of 0.2 M phosphate of pH 6.5 (anaerobic in quartz tube of 3 mm I.D.); ——— and ···· as in (A); ---- after addition of 1.7 μmole of DPNH at 0°.

Partial reduction of this enzyme resulted in the appearance of two absorption bands at long wave lengths (580 and 620 mμ). EPR signals and light absorption intensity altered in parallel as the enzyme was reduced stepwise

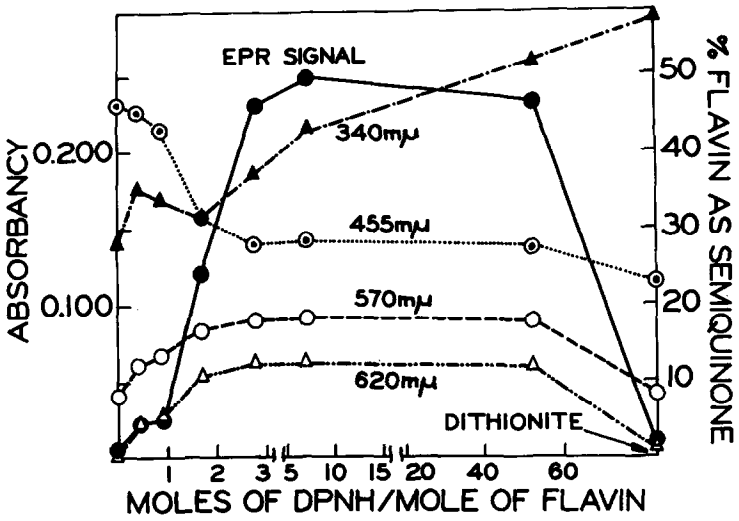

Fig. 3 Changes of reflectance and EPR signal (representing flavin semiquinone expressed as % of total bound flavin) during anaerobic titration of dihydroorotic dehydrogenase with DPNH at 0°. Data from experiment of Fig. 2B.

anaerobically, and also during subsequent reoxidation by air. Similar experiments performed with dihydroorate as the substrate gave identical results, except that higher substrate concentrations were required. The fact that the same absorption bands appear upon reduction of the enzyme with DPNH or dihydroorotate would appear to rule out flavin-substrate charge transfer complexes as a basis for the absorption at long wave lengths in the presence of substrates.

It is particularly noteworthy that even in the presence of 50 moles of DPNH (4.4 mM) per mole of enzyme flavin, we were unable to reduce the flavin moiety of the enzyme beyond the state of maximal semiquinone formation, which amounted to approximately 50% of the flavin present. It would appear therefore, that dihydroorotic dehydrogenase represents the first instance of a flavoprotein for which direct evidence of the identity of the long wave lengths absorbing, and the EPR signal producing species is at hand and for which reaction with substrate leads only to partial reduction with maximal formation of semiquinone. The experiments reported here obviously give no

information on the role of the semiquinone in the catalytic turnover of the enzyme, but it may be expected that in an enzyme of the reported properties only a shuttle between oxidized flavin and semiquinone form occurs during catalysis.

REFERENCES

1. Aleman, V., and Handler, P., (in preparation).
2. Beinert, H., J. Biol. Chem. 225, 465 (1957).
3. Beinert, H., and Sands, R. H., Biochem. Biophys. Res. Comm. 3, 41 (1960).
4. Beinert, H., and Lee, W., Biochem. Biophys. Res. Comm. 5, 40 (1961).
5. Beinert, H., and Sands, R. H., in "Free Radicals in Biological Systems," M. S. Blois et al. eds., Academic Press, New York, 1961.
6. Beinert, H., Federation Proc. 21, 48 (1962).
7. Beinert, H., Griffiths, D. E., Wharton, D. C., and Sands, R. H., J. Biol. Chem., in press (1962).
8. Bray, R. C., Pettersson, R., and Ehrenberg, A., Biochem. J. 81, 178 (1961).
9. Fridovich, I., and Handler, P., J. Biol. Chem. 233, 1578 (1958 a).
10. Fridovich, I., and Handler, P., J. Biol. Chem. 233, 1581 (1958 b).
11. Fridovich, I., and Handler, P., J. Biol. Chem. 236, 1836 (1961).
12. Fridovich, I., and Handler, P., J. Biol. Chem. in press (1962).
13. Greenlee, L., Fridovich, I., and Handler, P., (in preparation).
14. Rajagopalan, K. V., Fridovich, I., and Handler, J. Biol. Chem. 237, 922 (1962).
15. Rajagopalan, K. V., and Handler, P., Federation Proc. 21, 47 (1962).
16. Sands, R. H., and Beinert, H., Biochem. Biophys. Res. Comm. 3, 47 (1960).