

ON THE FUNCTION OF IRON IN DPNH CYTOCHROME c REDUCTASE\*

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Mahler and Elowe (1954) showed that their preparations of DPNH\*\* cytochrome c reductase contained iron and presented evidence that part of this iron is reduced by substrate and reoxidized by cytochrome c. Recent work by Massey (1957) made it doubtful whether the experimental approach of these authors was valid. We have reinvestigated the oxidation-reduction of cytochrome reductase--as a model of an electron transport enzyme which contains non-heme iron--by paramagnetic resonance (EPR) spectrometry.

The enzyme was prepared according to De Bernard (1957). After 48 hours dialysis against chelators it contained 7  $\mu$ moles of iron and 3  $\mu$ moles of flavin per g of protein. In this state the enzyme showed a distinct EPR signal at  $-100^{\circ}$  which has been previously observed with  $Fe^{3+}$  containing glasses (Sands, 1955) and with lyophilized methemoglobin. The intensity of the signal was not increased when an excess of ferricyanide was added. However, when DPNH (enzymatically reduced) was added, the signal disappeared. When 39 mg of reductase (270  $\mu$ moles of iron and 115  $\mu$ moles of flavin) were titrated anaerobically (Fig. 1), the size of the signal decreased 40% after addition of 13  $\mu$ moles of DPNH, 70% with an additional 13  $\mu$ moles and disappeared completely after an equal increment. The quantity of iron indicated by the original signal can therefore not exceed 78  $\mu$ moles. The iron signal

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\*\* DPNH and TPNH, reduced di- and triphosphopyridinenucleotides, respectively.

reappeared in its original size and shape when air was admitted or ferricyanide was added.

When after disappearance of the iron signal more substrate was added a free radical signal appeared (Fig. 1). This is assumed to indicate a flavin

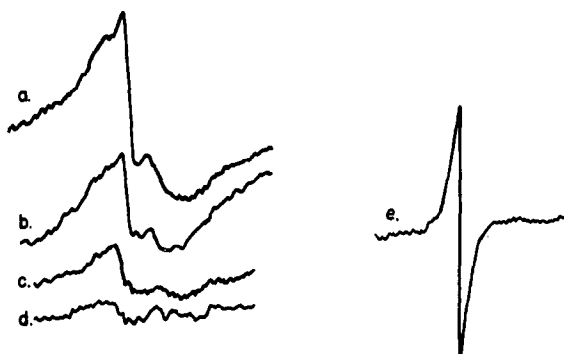


Figure 1. EPR signals of 39 mg of cytochrome reductase in 0.3 ml of 0.1 M tris acetate of pH 8.1 anaerobically at  $-100^{\circ}$ , (a) untreated ( $g=4.26(2)$ ), (b - d) DPNH added, (b) 13  $\mu$ moles, (c) 26  $\mu$ moles, (d) >39  $\mu$ moles, (e) radical signal ( $g=2.0031(2)$ , peak to peak width 20.9 gauss) after addition of 123  $\mu$ moles of DPNH.

semiquinone because flavins and flavoproteins form stable semiquinones under a variety of conditions and with reductants other than DPNH (Beinert, 1957)<sup>\*\*\*</sup>, whereas DPN does not readily form a one-electron intermediate (cf. Wallenfels and Gellrich, 1959). The radical concentration, estimated by comparison with FMN partly reduced at pH 0, increased as further increments of DPNH were added anaerobically and finally decreased when an excess of DPNH was present. Complete disappearance was never realized with the quantities of DPNH added (maximally 50 moles per mole of flavin). The free radical concentration increased again when either air was admitted or ferricyanide or DPN was added. Only after the radical signal had been completely abolished on reoxidation, the iron signal reappeared. DPN (25 moles per mole of reducible iron) did not restore the iron signal. The concentration of radical (% of total flavin present) was the following when the indicated total amounts of DPNH had been added: 39  $\mu$ moles, 0% (reduction of iron); 56  $\mu$ moles, 4%; 112

<sup>\*\*\*</sup> R. H. Sands and H. Beinert, unpublished.

$\mu$ moles, 18%; 123  $\mu$ moles, 23%; 179  $\mu$ moles, 19%; 235  $\mu$ moles, 15%; 200  $\mu$ moles of DPN, 23% (reoxidation); 2.2  $\mu$ moles of DPN, 40%. Anaerobically there was very little or no change of radical concentration, when the samples were incubated at 0° following additions. Aerobically a decrease of radical concentration was observed at 0° when the flavin was mostly in the oxidized state and an increase when the flavin was predominantly reduced. Otherwise the picture was very similar to that observed anaerobically and the maximal radical concentration was of the same order. The enzyme is about 50 times as active with DPNH as with TPNH. Accordingly, higher levels of TPNH were needed to reduce iron and flavin.

The following conclusions may be drawn from these experiments: All the iron in the enzyme which gives the observed signal changes in state on addition of DPNH. As the signal reappears on reoxidation the change probably consists in reduction. According to titration with DPNH the reducible iron is not more than 28% of the total found by chemical analysis. In the absence of measurements of initial rates our data do not permit us to derive the actual order of reduction or reoxidation of iron and flavin. At the times our measurements were made, 15-20 seconds after each addition, the iron was always found to be reduced before flavin and reoxidized after flavin. There was never any free radical seen unless the iron was completely reduced. However, the fact that DPN at an about 20-fold excess is able to reoxidize the flavin but not the iron suggests the sequence of electron flow: substrate  $\longrightarrow$  flavin  $\longrightarrow$  iron. It is a reasonable assumption that flavin is the component which interacts directly with the substrate and our observations are completely compatible with such a sequence, if the rate of reduction of flavin by substrate is slower than the rate of reduction of iron by flavin. Whereas the reduction of iron would thus be an obligatory sequel of flavin reduction, it is open to question whether iron is an obligatory intermediary in the interaction with all electron acceptors and in the reaction as it occurs in a more integrated system within mitochondria. It may, however, be recalled that Estabrook (1957) could not account, in

terms of known components, for all reducing equivalents added to a preparation of a more complex cytochrome c reductase.

Concerning the appearance of semiquinones during oxidation-reduction of the prosthetic flavin we conclude that a certain percentage of semiquinone, maximally about 40% at 50% reduction of the flavin, is in equilibrium with the oxidized and reduced forms of the prosthetic flavin at any intermediate oxidation state. The semiquinone appears similarly under aerobic as well as anaerobic conditions, so that reoxidation by dissolved oxygen is not the principal reaction leading to semiquinone formation. It is interesting that even at relatively high levels of substrate, semiquinone is still present indicating that reduction is not complete. This phenomenon may explain why specific spectral bands are found in "reduced" flavoproteins, which in fact may not be fully reduced according to the present findings.

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