Evidence for semiquinone-metal interaction in metal-flavoproteins.*

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When it was found that certain purified flavoproteins contained either iron or iron and molybdenum, the significance of these metal constituents became of great interest (DeRenzo et al, 1953; Green and Beinert, 1953; Richert and Westerfeld, 1954; Nicholas et al, 1954; Mahler and Elowe, 1954; Mahler et al, 1954; Singer et al, 1956; Friedmann and Vennesland, 1960). Their direct participation in the electron transport function of these enzymes could not be convincingly shown by chemical means (Mahler and Elowe, 1954; Singer and Massey, 1957; Massey 1958). With the aid of EPR spectroscopy, however, direct evidence was obtained, that the metal constituents in these proteins undergo changes during catalysis, which indicate valency changes (Mo(VI) $\rightleftarrows$ Mo(V); Fe(III) $\rightleftarrows$ Fe(II)), or at least major changes in the ligand environment of the metals (Palmer et al, 1964; Beinert et al, 1964; Rajagopalan et al, 1965; Aleman et al, 1965). Kinetic studies by EPR spectroscopy led to the conclusion that the sequence of electron carriers in two molybdenum-iron-flavoproteins is: substrate, Mo, flavin, Fe (Palmer et al, 1964; Rajagopalan et al, 1965). If this scheme is correct, then direct electron transfer between flavin and metal constituents might be expected.

In this context then, the mode of interaction of flavin and metal becomes of utmost interest. Flavin-metal interaction was most thoroughly studied in model systems by Hemmerich (1964) and Hemmerich et al (1963). These authors

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found that the oxidized and reduced forms of flavins have virtually no metal affinity (log K < 2), whereas the semiquinone is a metal chelator of appreciable strength (log K > 4) under physiological conditions. In the system

$$\text{flavin}_{ox} + \text{flavin}_{red} \rightleftarrows \text{semiquinone}$$

the equilibrium is therefore shifted toward the semiquinone state on addition of d-metal ions, as could be most directly shown by EPR spectroscopy for (diamagnetic) Zn^{2+} (Hemmerich et al, 1963) and Cd^{2+}.[1] In the presence of paramagnetic ions, such as Ni^{2+} or Fe^{2+}, however, the signal intensity was not increased beyond that of the metal-free system, while optical absorption changes and pH-titrations indicated metal-flavin interaction of the same magnitude as in the case of diamagnetic ions. It was suggested by Hemmerich et al (1963) that, in the case of paramagnetic ions, the EPR signal was not found in the semiquinone-metal chelates because of dipolar or lifetime broadening. In contrast to what is observed with free flavins, we observed that the addition of paramagnetic metal ions to partly reduced flavoproteins (free of endogenous metal) does not result in any changes of the optical spectrum. This excludes the possibility that added paramagnetic metal ions and protein-bound flavin semiquinones form the same stable chelates as do free flavin semiquinones. In the case of metal-flavoproteins, however, it appears likely that spectral changes observed on partial reduction include those due to the formation of semiquinone-metal chelates, but definite conclusions cannot be drawn yet as the changes are complex and not analyzed in detail. Again in contrast to the findings on free flavins (Hemmerich et al, 1963), EPR signals of flavin semiquinones are obtained in metal-containing as well as in metal-free flavoproteins upon partial reduction (g = 2). It is important to emphasize here, however, that in no case of the flavoproteins was an EPR signal observed of such intensity that it could account for the total flavin present. This would leave room for an appreciable portion of the flavin radical being engaged in a metal chelate devoid of EPR signal.

[1] A. Ehrenberg and P. Hemmerich, unpublished observations, communicated at the 6th International Congress of Biochemistry, New York, 1964.

While thus from spectral observations and considerations of total EPR intensity versus flavin concentration, no clearcut evidence for metal-flavin interaction in flavoproteins can be derived, an interaction does become evident from a comparison of the relaxation behavior of the free radicals formed in metal-containing and metal-free flavoproteins.

The relaxation rate of an unpaired spin system is characteristic of a particular environment of the unpaired electron and is generally much lower for organic free radicals than for metal ions, because of a stronger--and in some cases, considerably stronger--spin-orbit coupling in the latter case. Therefore, a change in relaxation rate indicates a change in the environment of the unpaired electron; an increase in rate would mean that an additional relaxation mechanism has become available in the system, and enhancement of the relaxation rate of a free radical in the presence of a paramagnetic metal ion finds a reasonable explanation in an interaction of radical and metal, which would allow the radical spin system to relax by coupling to that of the metal. Changes in spin relaxation are most readily detected by observing the saturation of the system with increasing incident electromagnetic radiation energy. Whereas at high frequencies (e.g., visible light, orbital excitation), I/I_o generally remains independent of the magnitude of I_o (Condon 1964), this is not so at low frequencies (e.g., microwave range, spin excitation), where I/I_o may increase with increasing I_o.

We have studied saturation of EPR absorption with increasing microwave power of radicals formed in metal-containing and metal-free flavoproteins and free flavins by addition of dithionite or substrate, where applicable. This work was done at low temperature (-170°) where no changes in radical concentration are likely to occur during measurement, signal to noise ratio is increased over that available at room temperature and saturation occurs at relatively low powers, so that the observation range is increased. Principally, the same behavior is observed at room temperature. Results are shown in Fig. 1. The square root of microwave power incident on the sample is plotted against signal

amplitude. In case of no saturation, a straight line through the origin should result.

At -170°, we could not find significant differences in the saturation

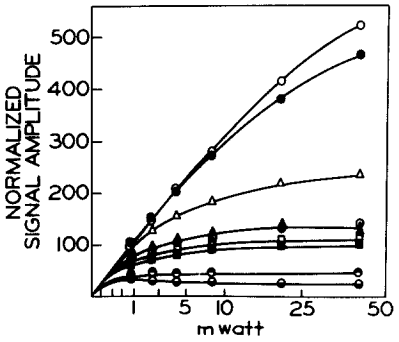

Fig. 1. Saturation with microwave power of EPR signals from flavin radicals at -170°. Signal amplitude in arbitrary units is plotted against the square root of microwave power (in mwatt) incident on the cavity. The numbers given on the abscissa refer to the values actually measured, i.e., before extraction of the square root. The markers on the left end of the abscissa correspond to 0.05, 0.1 and 0.25 mwatt, respectively. The signal amplitudes were normalized for all curves at 0.05 mwatt. The modulation amplitude was 3 gauss. The symbols refer to the following enzymes: —○—○— xanthine oxidase (s) (Palmer et al, 1964); —●—●— dihydroorotic dehydrogenase (s and d) (Friedmann and Vennesland, 1960; Aleman et al, to be submitted); —△—△— DPNH dehydrogenase (s) (Beinert et al, 1964); —□—□— aldehyde oxidase (s) (Rajagopalan et al, to be submitted); —⊙—⊙— succinic dehydrogenase (s) (Singer et al, 1956); —▲—▲— succinic Co enzyme Q reductase (s) (Ziegler and Doeg, 1962); —■—■— DPNH dehydrogenase (s) (Pharo and Sanadi, 1964); —◐—◐— lipoic dehydrogenase (s plus DPN); and —◑—◑— the metal-free flavoproteins and free flavin compounds (d). The letters "s" and "d" refer to the reductant used, substrate or dithionite, respectively.

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behavior of the free radicals of metal-free riboflavin, FMN and FAD and eight metal-free flavoproteins (Fig. 1): D- and L- amino acid (Massey et al, 1961; Singer and Kearney, 1950) and glucose (Kusai et al, 1960) oxidases, lactic oxidative decarboxylase (Sutton 1957), acyl CoA dehydrogenase (Crane et al, 1956), electron transferring flavoprotein (Crane and Beinert, 1956), microsomal TPNH cytochrome c reductase (Williams and Kamin, 1962) and a flavoprotein of unknown function from Azotobacter vinelandii (Shethna et al, 1964). The free radicals of lipoic dehydrogenase (Massey 1960) were saturated somewhat less readily. Saturation of the free radicals of metal-flavoproteins, on the other hand, differs widely and occurs in a range of considerably higher power than needed for

saturation of metal-free flavoprotein radicals. With the free flavin models, one has to avoid conditions giving rise to extensive contact of flavin radicals with each other, which provides an additional relaxation mechanism through exchange interaction. Thus, only concentrations < 10^{-4} M in radicals may be taken into consideration. In flavoproteins, this effect was not observed, which is readily explained by the steric separation of the active sites.

The saturation behavior of the free radicals of metal-free flavoproteins (glucose oxidase, acyl CoA dehydrogenase and azotobacter flavoprotein) in the presence of diamagnetic and paramagnetic metal ions was then studied. We observed no effect of added Zn^{2+} or Cd^{2+} ions at concentrations up to 0.1 M, whereas saturation was decreased on addition of paramagnetic metal ions such as Ni^{2+} or Fe^{3+} (Fig. 2). Ni^{2+} could be added in the form of simple hydrated salts whereas iron had to be added as the EDTA complex to prevent hydrolysis at the pH used (pH = 7). It can be seen from the figures that the quantity of added paramagnetic metal necessary to decrease the saturation to the level observed in metal-flavoproteins is approximately two orders of magnitude higher than that of the endogenous metal of metal-flavoproteins. It is also remarkable that iron added as the EDTA complex is still effective, where flavin could only temporarily occupy one coordination site of the iron. The question arises, therefore, as to wh

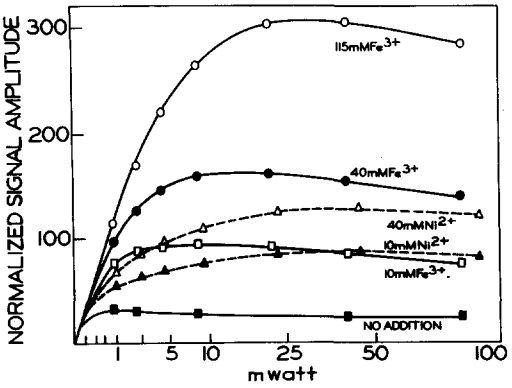


Fig. 2. Saturation curves of the radicals produced from glucose oxidase in the presence of increasing amounts of ferric EDTA and nickelous nitrate. Conditions and evaluation as for Fig. 1, but plotted with expanded abscissa.

might be the most important structural parameters determining the extent of spin relaxation in a system containing d-metal ion and organic radicals. To answer this question, we conducted preliminary studies on the saturation behavior of suitable models, e.g., the semiquinone cation of 1,3-Dimethyl-5-benzylleukolumiflavin (I, $R_1 = R_2 = R_3 = CH_3$, $R_4 = CH_2C_6H_5$) (Dudley et al, 1964). The optical and EPR spectra of this flavin radical derivative are essentially those of the FMN radical cation (Fig. 3, I, $R_1 = CH_2(CHOH)_3\ CH_2OPO_3H_2$, $R_2 = R_3 = R_4 = H$) (Dudley et al, 1964). It is, however, unable to form a metal complex of the type II, Fig. 3. Indeed, no effect of added paramagnetic metal ions on the saturation behavior of the semiquinone was observed. On the other hand, if $R_4 = H$, the metal present in solution can temporarily replace the protein at N(5) and interact with the unpaired π-electron. Accordingly, when $R_4 = H$, we found the relaxation rate to be increased upon addition of Ni^{2+}.

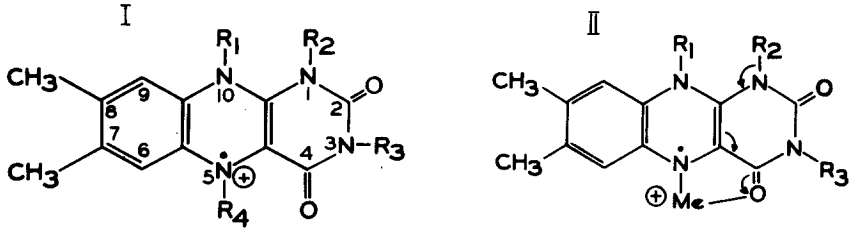


Fig. 3. Structure of flavin radical cation I and flavin radical-metal chelate II. If in II, $R_2 = H$, proton dissociation from this position will ensue.

This is in agreement with the findings of Ehrenberg, Eriksson and Hemmerich (1964) on spin distribution in flavin radicals, which show high spin density at N(5), whereas the probability of finding the unpaired electron in the pyrimidine part of the flavin nucleus is very low.

It would thus appear that we can classify the observed interactions into three general types: 1) The stable radical-metal complex (cf. Fig. 3, II), as it is formed from partially reduced free flavins and paramagnetic metal ions. In this case, characteristic changes in optical spectra, but no EPR signals are

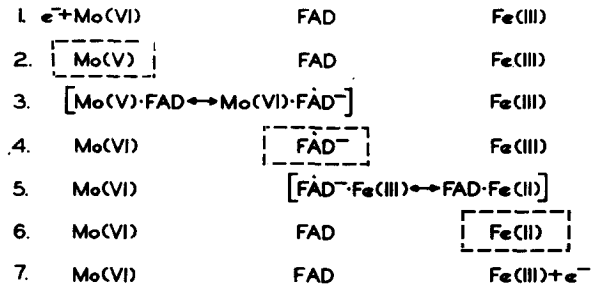
observed, and relaxation is probably very rapid. 2) The type of interaction observed with the flavin radical cation I, R_4 = H (Fig. 3) and paramagnetic metal ions or with flavoproteins when added or endogenous paramagnetic metals are present. In this case, the optical spectra do not change on addition of metal, EPR signals due to free flavin radicals are observed, but spin relaxation is substantially increased. A full explanation of this phenomenon remains the goal of further studies. At present, it appears most plausible to us that, in fact, radical-metal complexes similar to II (Fig. 3) are formed, but that these complexes are labile and hence, the equilibrium concentration of the free reactants in the system:

$$\text{radical} + \text{metal} \rightleftarrows \text{radical-metal-complex}$$

appreciable. EPR signals could, therefore, be observed from the free reactants, but the lifetime of the free radical species would be shortened by rapid oscillation of the proton at N(5) allowing and disrupting orbital overlap between the d-metal and the flavin π system. If the oscillations between these states were of a proper frequency, an effect upon spin relaxation would be expected, as observed. 3) The case of the non-coordinating radicals. Here, no radical-metal complex of any lifetime can be formed and no effect on relaxation rate is observed, suggesting that the flavin may have to enter the primary coordination sphere of the metal and to make an effective π and σ orbital overlap to experience an effect on spin relaxation. Apparently at the distances involved in this model, relaxation based solely on dipolar interactions is not detectable. The possibility has to be considered, however, that with special "conducting" ligands, e.g., imidazole, spin relaxation through the metal may occur at a greater distance. This is under current investigation.

On the basis of these results, we suggest that semiquinone-metal complexes are, in fact, involved in electron transfer in metal-flavoproteins, although, under certain conditions, there appear to be appreciable equilibrium concentrations of "free" flavin radical and metal (each, of course, bound to the protein in its specific way). The following scheme summarizes this for the example of a molyb-

denum-iron-flavoprotein. This scheme is primarily designed to express the relaxation data reported here and will eventually have to be combined with information obtained by other approaches (Palmer et al, 1964), to furnish a more complete picture of the interactions. The species shown within the dashed frames are those that are detectable by EPR.



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