New evidence for the occurrence of free radicals in flavoprotein catalysis

Although earlier work by various investigators[1-7] has left little doubt that riboflavin passes through an intermediate semiquinoid oxidation state on reduction and reoxidation, little is known about the significance of such intermediates in flavoprotein catalysis. HAAS[8] reported in 1937 on a red intermediate which he obtained on partial reduction of the old yellow enzyme in presence of TPN, but this interesting observation has remained isolated. In fact, the belief has taken hold more recently[9] that flavoproteins are unable to carry out one-electron transfer efficiently unless they contain an auxiliary component.

Evidence will be presented here which favors the concept that intermediates of semiquinoid nature may indeed occur at significant concentrations during flavoprotein catalysis. This evidence rests on observations with the three fatty acyl CoA dehydrogenases from pig liver[10-12]. Only one of these enzymes contains significant amounts of a metal[10]. Otherwise, these flavoproteins have very similar properties. The phenomena, which will be discussed here, were observed with all three enzymes and were most thoroughly studied with the fatty acyl CoA dehydrogenase most active with substrates from C_8 to C_{12}.

When substrate is added to this yellow enzyme, which will be designated as Y, the light absorption at 450 mμ is only partly abolished and a new broad band appears between 500 and 650 mμ which imparts a brownish-green color to the enzyme. The same band is observed when Y is reduced by excess dithionite in presence of the oxidized form of the substrate, *i.e.*, α,β-unsaturated fatty acyl CoA. On the basis of the assumption that only the fully reduced form of Y, designated as YH_2, can exist in presence of excess dithionite, it was suggested[13] that this new band indicates a complex of reduced enzyme and oxidized substrate. A similar conclusion was recently drawn by DOLIN[14] who observed an absorption band of the same characteristics and under corresponding conditions with the flavoprotein DPNH peroxidase from *S. faecalis**.

That this earlier suggestion could not be correct, became apparent when the characteristic band was also observed during reduction of Y by dithionite in absence of any substrate. The band appeared only during the few seconds in which the flavin absorption at 450 mμ faded from its maximum to its minimum and it was most pronounced when the absorption at 450 mμ was halfway between maximum and minimum values. It became then visible again, while YH_2 was being reoxidized by air. These transient phenomena were recorded with a movie camera from the screen of a rapid scanning spectrophotometer which operates with a cathode ray tube**. With this device it was then possible to detect the same band on reduction or reoxidation of free flavin. The following observations show that dithionite has no specific function beyond its role as reducing agent and that the only specific component which is necessary for the phenomenon to occur, is flavin: the band can be produced on (1) reduction of Y by dithionite in absence of substrate, (2) reduction of free flavin by dithionite, (3) reduction of Y with substrate in absence of dithionite, (4) reduction of free flavin by sodium amalgam, (5) reoxidation of YH_2 by O_2, and (6) reoxidation of reduced free flavin by O_2.

The conditions described above, under which the new absorption band appears, definitely point to an intermediate oxidation stage as the basis of the phenomenon. Intermediate oxidation stages of riboflavin and related compounds have been described some twenty years ago[1-7] and good evidence was adduced that they are of semiquinoid, *i.e.*, free radical nature. However, an intermediate state of oxidation can also be represented by a quinhydrone-like dimer of oxidized and reduced forms. Criteria for the differentiation of such a dimer and a free radical monomer have been discussed by MICHAELIS[6,16]. The effects of concentration and temperature changes were used here to differentiate between free radical and dimer.

The difference spectrum of the intermediate at full development and the oxidized form is shown in Fig. 1 for FAD, reduced by dithionite (curves 1 and 2), and for enzyme, reduced by substrate (curve 3). Curves 2 and 3 were recorded at 0.5 cm light path, curve 1 at 10 cm light path, such that the total amount of FAD in the light path was identical for curves 1 and 2 and was about 20 times lower for the enzyme (curve 3). It is evident that there is an additional band in the infrared when the concentration of FAD is $4 \cdot 10^{-3} M$. This infrared band is almost eliminated when the concentration of FAD is down to $2 \cdot 10^{-4} M$ and it is absent with the enzyme at $2 \cdot 10^{-4} M$ concentration of flavin. The effect of temperature on the infrared band is marked; a record of the spectrum at 4° shows an infrared band even higher than that of curve 2, whereas a record at 49° is almost identical with curve 1. The band in the infrared with $\lambda_{max.}$ 900 mμ is therefore tentatively assigned to a dimer (or less likely a higher polymer), whereas the band with $\lambda_{max.}$

* MAHLER AND ELOWE had also reported the appearance of a similar band on reduction of DPNH cytochrome *c* reductase[15].

** Rapid scanning spectrophotometer, American Optical Company, Buffalo, New York.

570 mμ is assigned to the concentration- and temperature-independent monomeric free radical. In curve 2 the radical peak is higher than in curve 1, although an appreciable amount of the radical would be expected to have dimerized. This can be attributed to a considerable overlapping of the absorption of the dimer with that of the radical which adds to the total absorption at 570 mμ.

The absorption bands attributed to the radical and the dimer are dependent on pH. MICHAELIS, *et al.*[5,6] have deduced pK values for the free radical of riboflavin (pK 1.3 and 6.5) and it is of interest that the bands observed with free flavin in the present work show their maximum shift near these pK values. Below pH 1 the brownish-red intermediate of KUHN AND WAGNER-JAUREGG[1] ($\lambda_{max.}$ 475 mμ) is obtained. Between pH 2 and 7 a brownish-green intermediate appears ($\lambda_{max.}$ 570 mμ) and at higher pH values no specific band attributable to the free radical was found although a slight absorption in the region of 600 mμ and the band indicating the dimer remain. The maxima of the dimer-band are the following: pH < 1 to 6, 850 mμ; pH 8 to 10, 1050 mμ; pH 11 to 12, 830 mμ. The fact that there is a shift between pH 10 and 11 indicates an additional dissociation for which there is no evidence from the spectrum of the radical and from MICHAELIS' earlier work.

A comparison of the concentrations of free and enzyme-bound flavin and the height of the 570 mμ peak in Fig. 1 shows that the amount of intermediate formed from enzyme-bound FAD is about 20 times as high as that formed from free FAD, if one assumes an identical molar extinction coefficient for both forms of FAD. Photographic records have shown that the absorption of the substrate- and dithionite-produced intermediate of Y is essentially the same. However, the intermediate is stable for many hours when produced by addition of substrate, whereas it vanishes within seconds when produced by dithionite in absence of substrate. It may therefore be concluded that com bination of the enzyme with the substrate stabilizes the intermediate.

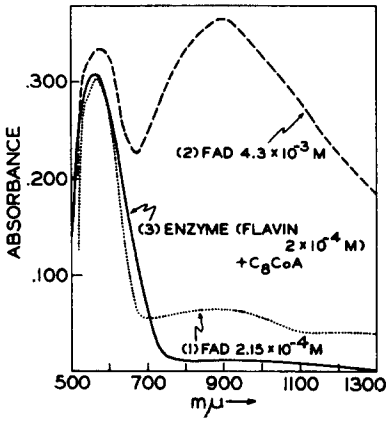
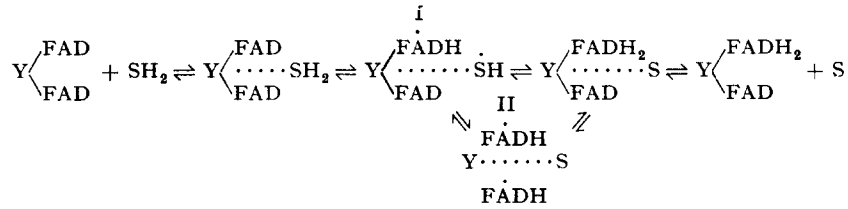


Fig. 1. Difference of spectrum of FAD and Y at intermediate oxidation state minus that of FAD and Y at fully oxidized state, replotted from tracings of Beckman recording spectrophotometer, Model DK 1. Curve 1: $2.15 \cdot 10^{-4} M$ FAD, reductant $Na_2S_2O_4$, light path 10 cm. Curve 2: $4.3 \cdot 10^{-3} M$ FAD, reductant $Na_2S_2O_4$, light path 0.5 cm. Curve 3: Fatty acyl CoA dehydrogenase Y, containing $2 \cdot 10^{-4} M$ bound FAD, reductant octanoyl CoA, light path 0.5 cm. FAD in $0.17 M$ citrate, pH 6.1; Y in $0.1 M$ phosphate, pH 7.0. Because of the higher absorption of the oxidized forms at 450 mμ the difference spectra become all negative at about 500 mμ.

This conclusion is further supported by the inertness of the flavoprotein in presence of substrate towards oxidizing or reducing agents. It has been reported[11,13] that the enzyme, when partially reduced by substrate is not oxidizable by many commonly used electron acceptors and there is also little further reduction by dithionite. FAD bound to these proteins seems inaccessible to the mentioned agents. Even when substrate, *e.g.* octanoyl CoA, is added to Y in amounts less than stoichiometric to the FAD of the enzyme, neither oxygen nor 2,6-dichlorophenol indophenol nor ferricyanide will reoxidize the partially bleached Y instantaneously—that is, the enzyme is not able to turn over even once. Ferricyanide will reoxidize YH_2 within about 10 minutes, quinones and phenazine methosulfate at an even faster rate. A specific flavoprotein, which has been called the electron-transferring flavoprotein (ETF) and which has been described recently[17], is needed to break the enzyme-substrate combination and make the FAD groups of Y available for oxidative attack by those agents which are unable to reoxidize YH_2.

It has been shown in anaerobic experiments that Y, when partly reduced with suboptimal amounts of dithionite in absence of substrate, is instantaneously reoxidized by O_2, indophenol and ferricyanide. This shows that there is no basic difficulty in reoxidation of this enzyme either by one- or by two-electron acceptors. In fact the one-electron acceptor ferricyanide oxidizes YH_2 slowly in presence of substrate and absence of ETF, whereas indophenol is inert.

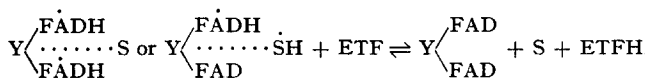
The interactions of Y and substrate may be visualized in the following way, where FAD and $FADH_2$ signify the oxidized and reduced forms, respectively, of the enzyme-bound flavin and S and SH_2 the oxidized and reduced forms of the substrate:

(1) 

The intermediate complexes I or II are thought to stabilize the flavin radical. Y has been shown to have 2 moles of FAD per mole, so that formation of II would not necessitate interaction of more than one enzyme molecule with one molecule of substrate. Stabilization of the radical in complex II would also circumvent the postulate of a stable free radical form of the substrate $\cdots\dot{S}H$ as written in (1), for which there is no direct evidence. The fact that oxidizing and reducing agents interact only very sluggishly with the equilibrium system of reacting species tentatively represented by scheme (1) indicates that in the presence of substrate extremely small amounts of the enzyme are in the form of free oxidized or reduced Y. It was pointed out above that the free forms of the enzyme are readily reducible and oxidizable. It is therefore unlikely that the attack of ETF is confined to the reoxidation of free reduced Y according to (2). FAD and $FADH_2$ indicate enzyme-bound flavin:

(2) $$FADH_2 + ETF \rightleftharpoons FAD + ETFH_2$$

It is more likely that ETF may intervene at the intermediate stage I or II:

(3) 

ETF has been shown to carry out one electron transfers readily.

These studies will be extended to other flavoproteins. It is also planned to confirm the conclusion that the intermediates concerned are indeed free radicals by independent physical methods.

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