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Light-Mediated Reduction of Flavoproteins with Flavins as Catalysts†

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ABSTRACT: It has been found that small amounts of free flavins greatly accelerate the photochemical reduction of flavoproteins both to the radical and fully reduced oxidation states. This catalytic effect has been shown to be due to the rapid photochemical reduction of the free flavin to its fully reduced state, followed by its reaction with the flavoprotein to yield flavoprotein radical and by its reaction with flavoprotein radical to yield fully reduced flavoprotein. Evidence is presented that the same route may occur with flavoproteins in the absence of added flavins. In this case the photoreduction is mediated by the small equilibrium concentration of free flavin coenzyme present in a flavoprotein solution. Hence, it is suggested that flavoprotein reduction with EDTA as photosubstrate does not involve an excited state of the holoprotein, nor contact of EDTA with the enzyme, but exchange of electrons between enzyme flavin and free reduced flavin.

The facile photoreduction of free flavins by a variety of amino acids, carboxylic acids, and amines has been known for many years (Frisell et al., 1959). One of the most effective photoreductants is ethylenediaminetetraacetic acid (EDTA)[1] (Frisell et al., 1959; Vernon, 1959). Under anaerobic conditions, over a wide pH range, photoreduction of flavins by 10 mM EDTA is accomplished in a matter of seconds with a source of white light such as that given by commercial slide projectors or "sun-guns", according to eq 1.

$$Fl_{ox}* + EDTA \xrightarrow{H_2O} Fl_{red}H_2 + ED\text{-triacetate} + CH_2O + CO_2 \quad (1)$$

$Fl_{red}H_2$ could be generated by any of four possible routes: (1) direct $2e^-$ reduction, i.e., hydride transfer; (2) direct $1e^-$ reduction, followed by flavin radical dismutation; (3) flavin radical reduction by substrate radical; (4) protolysis of a preformed flavin-substrate "adduct" R-Fl_{red}H. (In the present case, EDTA ≡ $RCOO^-$.)

Pathway 1 can be neglected, since EDTA is no hydride donor. In fact a C-COO^- bond is split preferably during its flavin-sensitized photooxidation, rather than a C-H bond, as shown by the formation of carbon dioxide and formaldehyde (Frisell et al., 1959; Penzer and Radda, 1968; Haas and Hemmerich, 1972). There is one piece of evidence for CH breakage and, consequently, keto acid formation, namely, the data of Enns and Burgess (1965) on EDTA photooxidation by flavin in the absence of oxygen. Even in this case, however, quantitative formation of CO_2 was found to occur (V. Massey, unpublished data). Glyoxylate formation must, therefore, be a secondary process.

Hence, while some flavosemiquinone is indeed formed, as seen in flash photolysis experiments done at first by Holmström (1964) and recently by Knappe et al. (1977; W. R. Knappe, P. Hemmerich, H. J. Duchstein, and H. Fenner, to be published), the amount of Fl_{ox} bleached per flash exceeds the amount of radical formation. This can only be explained by assuming that pathway 4 is the prevailing reaction. In 1967 we reported on photoreaction of flavins with a variety of carboxylic acid salts. With several of these, notably phenyl acetate, the product of the photoreaction was not 1,5-dihydroflavin, but stable N(5)- and C(4a)-benzyl adducts of dihydroflavin, with stoichiometric release of 1 mol of CO_2 per mol of flavin alkylated (Hemmerich et al., 1967; Walker et al., 1967). With the closely related compound, mandelate, no stable adducts were formed, the products of the photoreaction being 1,5-dihydroflavin, benzaldehyde, and CO_2 (Walker et al., 1970). In view of the similarity of the starting reactants and the stoichiometric release of CO_2 observed also for EDTA (V. Massey, unpublished results), it seems reasonable to assume that the major difference between the two photoreactions is in the differing stability of intermediate adducts (Walker et al., 1970). That intermediate flavin adducts are indeed formed with EDTA was shown by Elliot and Bruice (1973) in the case of two flavin derivatives. Haas and Hemmerich (1972) have

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[1] Abbreviations used; fl_{ox}, $FlH^\cdot$, and $Fl_{red}H_2$, the oxidized, semiquinoid, and fully reduced forms of flavin; EFl_{ox}, $EFlH^\cdot$, and $EFl_{red}H_2$, the corresponding forms of enzyme-bound flavin; EDTA, ethylenediaminetetraacetate; RBP, riboflavin-binding protein.

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pointed out that amino acid anions rapidly react by decarboxylation in preference to dehydrogenation, while the neutral form is inert in the dipolar state, with only the tautomer $RCH(NH_2)COOH$ slowly undergoing CH cleavage.

In contrast to the facile photoreduction of free flavins by EDTA to the 1,5-dihydroflavin, photoreaction of flavoproteins with EDTA occurs only slowly and in most cases gives quantitative or near-quantitative formation of the flavoprotein radical (Massey and Palmer, 1966), with the fully reduced enzyme being in general obtained only on prolonged light irradiation. The first step is nondestructive, and since its introduction the method has been employed widely for the production of flavoprotein semiquinones. The prolonged exposure to light, as required for generation of the fully reduced flavoprotein, may, however, damage the proteins. In general it has been found that a particular apoenzyme stabilizes either the blue neutral semiquinone or the red anionic semiquinone over the whole range of pH stability of the enzyme, with flavoprotein "oxidases" stabilizing the radical anion, and "dehydrogenases" stabilizing the neutral radical (Massey et al., 1969b). There are a few known exceptions, where either type of flavin radical is stabilized depending on the pH. Thus glucose oxidase yields either type, with a pK at pH 7.5 (Massey and Palmer, 1966). Another example is lysine monooxygenase, with a pK in the neutral pH region (Flashner and Massey, 1974). With free flavins the pK of the neutral-anion radical transition is 8.3–8.6 (Ehrenberg et al., 1967; Land and Swallow, 1969; Draper and Ingraham, 1968).

Various explanations have been put forward for the great differences in radical yields between free flavins and flavoproteins, and the presumed differences in the route of photoreaction. With free flavins it is known that there exists a very rapid equilibrium between the oxidized and reduced forms and radical, with the equilibrium lying overwhelmingly against radical accumulation (eq 2) (Gibson et al., 1962; Swinehart, 1966; Ehrenberg et al., 1967).

$$Fl_{ox} + Fl_{red}H_2 \rightleftarrows 2FlH^{\circ} \quad (2)$$

In the case of flavoproteins, the experimentally observed high yield of radical would imply that such an equilibrium with protein-bound flavins would have to lie strongly to the right (eq 3).

$$EFl_{ox} + EFl_{red}H_2 \rightleftarrows 2EFlH^{\circ} \quad (3)$$

Hence, we have considered for a while that the EDTA–light-mediated production of flavoprotein radicals might proceed through the rate-determining production of $EFl_{red}H_2$ (in analogy to the above pathway 4 of $Fl_{red}H_2$ formation in the free state) followed by rapid coproportionation with remaining EFl_{ox}. This possibility can be excluded for intermolecular reactions between oxidized and reduced flavoproteins, since many examples are documented where such interaction is extremely slow (see Mayhew and Massey, 1973). The possibility cannot be excluded in the case of dimeric or multimeric flavoproteins, where in principle intramolecular electron transfer may occur between reduced flavin of one protomer and oxidized flavin of another. While such interflavin electron transfer might have catalytic importance with some enzymes, the evidence to be presented in this paper makes it unlikely to be of general importance in the photochemical generation of flavoprotein radicals.

In 1967, McCormick et al. observed that the EDTA-mediated photoreduction of D-amino acid oxidase was significantly stimulated by added FAD and FMN. It was claimed that no such stimulation was observed with glucose oxidase and the results were interpreted as being due to the binding of free $FMNH_2$ and $FADH_2$ by D-amino acid oxidase followed by intramolecular reduction of the enzyme-bound FAD to produce semiquinone. We have investigated a wide range of flavoproteins and found that in all cases (including glucose oxidase) there is marked stimulation in the photochemical reduction by even trace amounts of added flavins. To eliminate complications of specific binding we have avoided the use of the natural coenzyme flavins FMN and FAD and have used instead model flavins such as tetraacetylriboflavin, 3-methyllumiflavin and lumiflavin 3-acetate. The catalytic effects of these flavins have been shown to be due to their rapid dark reaction in the reduced form with oxidized flavoproteins (reactions 4–6).

$$Fl_{ox} \xrightarrow[h\nu]{EDTA} Fl_{red}H_2 \quad (4)$$

$$Fl_{red}H_2 + EFl_{ox} \xrightarrow{dark} FlH^{\circ} + EFlH^{\circ} \quad (5)$$

$$Fl_{red}H_2 + EFlH^{\circ} \xrightarrow{dark} FlH^{\circ} + EFl_{red}H_2 \quad (6)$$

By the rapid disproportionation reaction (eq 2) the free flavin radicals produced re-form Fl_{ox}, accounting for their effectiveness in catalytic amounts.

Evidence is also presented that, in the case of photoreduction of flavoproteins in the absence of added flavin, the comparatively slow photoreaction is mediated to a large extent by the trace amounts of free coenzyme in equilibrium with apoprotein (eq 7)

$$EFl_{ox} \rightleftharpoons E + Fl_{ox} \quad (7)$$

Materials and Methods

Flavoproteins. These were prepared by standard literature methods; *Peptostreptococcus elsdenii* flavodoxin (Mayhew and Massey, 1969), pig kidney D-amino acid oxidase (Curti et al., 1973), *Crotalus adamanteus* L-amino acid oxidase (Wellner and Meister, 1960), *Aspergillus niger* glucose oxidase (Swoboda and Massey, 1965), pig heart lipoyl dehydrogenase (Massey et al., 1960), egg white flavin-binding protein (Blankenhorn et al., 1975; Becvar, 1973), *Mycobacterium smegmatis* L-lactate monooxygenase (Sullivan, 1968), *Pseudomonas spp.* melilotate hydroxylase (Strickland and Massey, 1973), *Pseudomonas fluorescens p*-hydroxybenzoate hydroxylase (Entsch et al., 1976), spinach ferredoxin-$NADP^+$ reductase (Shin et al., 1963), Old Yellow Enzyme (Abramovitz and Massey, 1976), milk xanthine oxidase (Massey et al., 1969a).

Flavins. Riboflavin and flavin mononucleotide (FMN) were gifts of Hoffmann-La Roche & Co., Basle. The riboflavin was chromatographically pure, the FMN contains approximately 10% riboflavin and unknown constituents (Massey and Swoboda, 1963; Scola-Nagelschneider and Hemmerich, 1976). The other flavins used were chromatographically pure and were prepared by standard literature methods; tetraacetylriboflavin (Scola-Nagelschneider and Hemmerich, 1976; Hemmerich et al., 1960), 3-methyllumiflavin (Hemmerich, 1964; Hemmerich et al., 1960), lumiflavin 3-acetate (Hemmerich, 1964).

Other Chemicals. Ethylenediaminetetraacetic acid (EDTA) was obtained from Mallinkrodt or from Merck. All other reagents used were of analytical grade. All solutions were prepared with glass-distilled water.

Photochemical Reductions. Flavoprotein solutions were

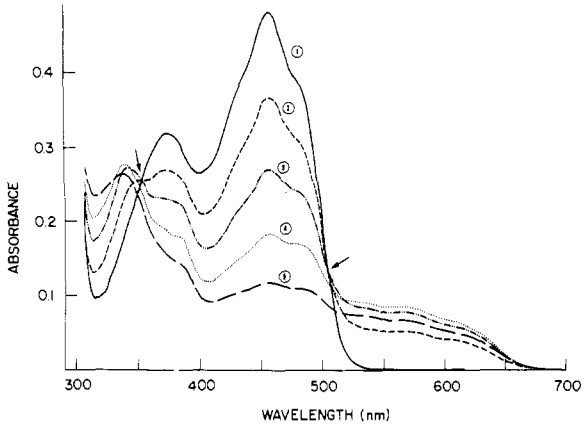

FIGURE 1: Photoreduction of egg white riboflavin-binding protein. Conditions: 50 μM riboflavin, 56 μM riboflavin-binding protein, 0.06 M phosphate, pH 7.0, 15 mM EDTA, temperature 25 °C. (Curve 1) Before illumination; (curves 2-5) after 8 min, 23 min, 60 min, and 240 min illumination. The arrows show isosbestic points (353 and 504 nm) in the early stages of reduction. After irradiation air was admitted and the original spectrum was regained rapidly.

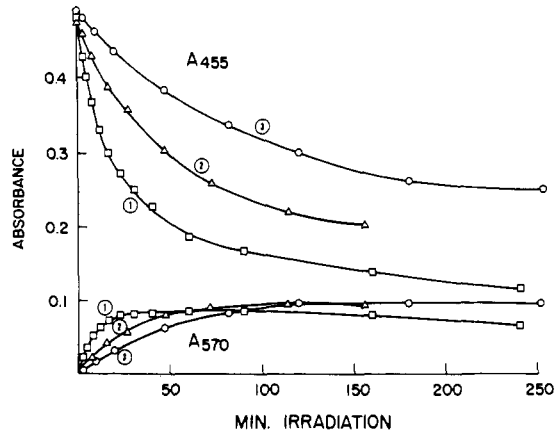

FIGURE 2: Effect of the concentration of riboflavin-binding protein on the rate of photoreduction. Conditions as in Figure 1, except the concentration of RBP was varied, being 56 μM in curve 1, 70 μM in curve 2, and 92 μM in curve 3.

dialyzed vs. appropriate buffers or subjected to gel filtration with Sephadex G-25 before use. Solutions contained in anaerobic spectrophotometer cells equipped with side arms were made anaerobic by repeated cycles of evacuation and flushing with O_2-free N_2. Added flavins and EDTA are conveniently stored in the side arms during this process, and then mixed with the enzyme before light irradiation is carried out. The anaerobic cuvette was placed in a water bath maintained at the desired temperature and illuminated with a sun gun (Smith-Victor Corp., Griffith, Ind.) at a distance of 7 cm from the sample. The Pyrex glass water bath transmits light only at wavelengths greater than 320 nm. The intensity of illumination was controlled by a rheostat. Except where otherwise noted a standard light intensity of approximately 8×10^6 ergs per cm^2 per s (estimated with a Yellow Springs Model 65 radiometer) was employed throughout. After each period of illumination any dark changes in absorbance were allowed to come to completion before the absorption spectrum was recorded, using either a Cary 17 or 118 recording spectrophotometer.

Stopped-Flow Studies. Reduced flavins were prepared under anaerobic conditions in gas-tight tonometers by light irradiation of the oxidized flavin in the presence of 10 mM EDTA. Reduction is monitored readily by loss of the intense fluorescence of the oxidized flavin. The reduced flavins were transferred under positive pressure of N_2 to the stopped-flow apparatus and reacted with anaerobic solutions of the oxidized flavoproteins studied. The stopped-flow apparatus used was a Gibson-Milnes (1964) instrument, equipped with an internally calibrated logarithmic operational amplifier, with data collected by a transient recorder (Transidyne General Corp., Ann Arbor, Neurograph Model N-3) and then transferred to graph paper with an X-Y recorder. We are indebted to Dr. D. P. Ballou and Mr. G. Ford for these modifications.

Results and Discussion

Photoreduction of Egg White Flavin Binding Protein in the Presence of Different Flavins. The riboflavin-binding protein of egg whites (RBP) has been found to bind avidly riboflavin and a variety of simple model flavins. The kinetic and thermodynamic constants for such binding have been determined by Becvar (1973). When the RBP-riboflavin complex is irradiated with visible light anaerobically in the presence of EDTA, the typical absorption spectrum of a neutral flavin radical is produced slowly followed by its even slower conversion to the fully reduced flavin form (Figure 1). It was noted that the rate of the photoreduction was influenced markedly by the molar ratio of RBP and riboflavin (Figure 2). Under the conditions used, pH 7.0, 25 °C, the K_d of riboflavin binding was determined by Becvar (1973) to be 1.2×10^{-9} M. Thus, with the molar excesses of RBP over riboflavin employed in the experiments of Figure 2, it is evident that less than 1/1000 of the added riboflavin can be in the free state in the equilibrium

$$\text{RBP} + \text{RF} \rightleftharpoons \text{RBP} \cdot \text{RF}$$

Nevertheless, the photoreduction rate is reasonably well correlated with the calculated concentration of free riboflavin (Table I), suggesting that it is the latter which controls the rate of the photoreduction. This conclusion is borne out when the photoreaction is carried out with RBP in complex with 3-methyllumiflavin (Figure 3) and lumiflavin 3-acetate (results not shown). The K_d values for these flavins are 5.05×10^{-8} M and 4.55×10^{-7} M, respectively (Becvar, 1973). Hence, at comparable concentrations of riboflavin binding protein, considerably more free flavin would be expected in equilibrium, and considerably faster photoreduction would be expected if the rate of photoreduction were indeed controlled by the free flavin concentration. Table I shows a quantitative treatment of the results. Within each set there is nearly constant "second-order" rate of reduction to the neutral semiquinoid form of RBP, when the observed first-order rate of reduction is divided by the estimated equilibrium concentration of free flavin. In addition, there is comparatively little variation of this value with the different flavins used. Although the observed half-times of semiquinone production vary by a factor of 170, the value for k_{obsd}/[free flavin] varies by only a factor of 3. These results suggested strongly that the EDTA-mediated photoreduction of flavoproteins may occur via the small equilibrium concentration of free flavin coenzyme, which would be rapidly photoreduced, and then in subsequent dark reactions react with holoenzyme to produce semiquinone, and with semiquinone to produce fully reduced enzyme (reaction sequence 7, 4-6, 2)

$$\text{EFl}_{ox} \rightleftharpoons \text{E} + \text{Fl}_{ox} \qquad (7)$$

$$\text{Fl}_{ox} \xrightarrow[h\nu]{\text{EDTA}} \text{Fl}_{red}\text{H}_2 \qquad (4)$$

TABLE I: Effect of Equilibrium Concentration of Free Flavin on the Photoreduction Rate of Riboflavin Binding Protein.[a]

Flavin	K_d (M)	(RBP) total (M)	$(flavin)_{eq}$ (M)	$t_{1/2}$ (min)	k_{obsd} (min^{-1})	$k_{obsd}/(flavin)_{eq}$ (M^{-1} min^{-1})
Riboflavin	1.2×10^{-9}	5.6×10^{-5}	1×10^{-8}	7	9.9×10^{-2}	9.9×10^{6}
		7.0×10^{-5}	3×10^{-9}	20	3.47×10^{-2}	1.04×10^{7}
		9.2×10^{-5}	1.4×10^{-9}	34	2.04×10^{-2}	1.46×10^{7}
3-Methyllumiflavin	5.05×10^{-8}	1.0×10^{-4}	5×10^{-8}	0.733	0.945	1.89×10^{7}
		1.33×10^{-4}	3.04×10^{-8}	1.17	0.592	1.95×10^{7}
		2.0×10^{-4}	1.68×10^{-8}	2.47	0.280	1.67×10^{7}
Lumiflavin 3-acetate	4.55×10^{-7}	9.2×10^{-5}	5.42×10^{-7}	0.2	3.46	6.4×10^{6}
		1.23×10^{-4}	3.12×10^{-7}	0.33	2.08	6.7×10^{6}
		1.85×10^{-4}	1.68×10^{-7}	0.60	1.15	6.8×10^{6}

[a] Experimental conditions are given in the legends of Figures 2 and 3. The equilibrium concentrations of free flavins were calculated from the K_d values of Becvar (1973). The half-time of production of neutral semiquinone was used to calculate k_{obsd}. In all cases the total concentration of flavin was 5×10^{-5} M and the total concentrations of RBP as shown.

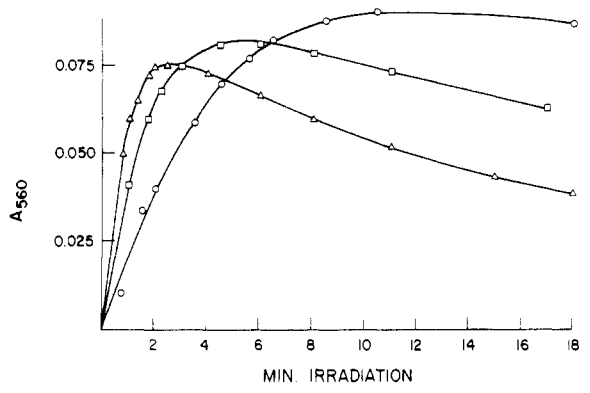

FIGURE 3: Effect of RBP concentration on the photoreduction rate when 3-methyllumiflavin was the flavin bound. Conditions as in Figures 1 and 2. The concentration of 3-methyllumiflavin was 50 μM; the concentrations of RBP were 100 μM (△), 133 μM (□), and 200 μM (○).

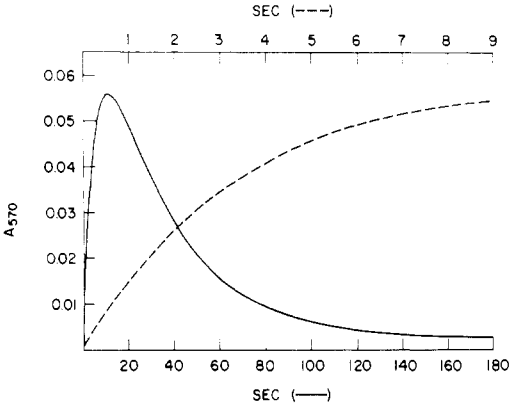

FIGURE 4: Reaction of glucose oxidase with reduced lumiflavin 3-acetate. Glucose oxidase, made anaerobic as described in Materials and Methods, was reacted in the dark with reduced lumiflavin 3-acetate in a stopped-flow spectrophotometer. The concentrations, after mixing, were 10.45 μM glucose oxidase and 100 μM reduced lumiflavin 3-acetate. Conditions: 0.12 M phosphate–0.05 M citrate, pH 5.3, 25 °C. The formation and disappearance of the glucose oxidase neutral radical were followed at 570 nm. The absorbance values shown are for the 2-cm light path of the stopped-flow apparatus. Thus the maximum extinction change observed was 2700 M^{-1} cm^{-1}. Based on the previously reported value for the semiquinone of 4000 M^{-1} cm^{-1} (Massey et al., 1970), it can be estimated that 67.5% radical was produced at the maximum. This corresponds well with the expected 75% for an A → B → C sequence in wich the rate of conversion A → B is 9× that of the conversion B → C, and B is the species being measured (Massey and Curti, 1967).

$$Fl_{red}H_2 + EFl_{ox} \xrightarrow{dark} FlH^\circ + EFlH^\circ \quad (5)$$

$$Fl_{red}H_2 + EFlH^\circ \xrightarrow{dark} FlH^\circ + EFl_{red}H_2 \quad (6)$$

$$2FlH^\circ \rightleftharpoons Fl_{ox} + Fl_{red}H_2 \quad (2)$$

In this hypothesis, the yield of flavoprotein semiquinone would be determined largely by the relative rates of steps 5 and 6, and the overall rate of photoreduction by the equilibrium concentration of free Fl_{ox} in step 7. If $k_5 \gg k_6$, almost quantitative flavoprotein radical yields would be expected. The closer both rates come together the easier it is to get full reduction. It must, however, be kept in mind that flavin is an ambiguous system which can transfer single electrons as well as $2e^-$ equivalents. This latter exchange between Fl_{ox} and $Fl_{red}H_2$ occurs possibly by way of formation of a very short-lived covalent bond—a process which may or may not involve additional π-charge transfer between the two flavin halves (Favoudon and Lhoste, 1975; Hemmerich, 1977). Hence, we cannot exclude the competitive pathway of direct full reduction of the enzyme as given in eq 8:

$$Fl_{red}H_2 + E\text{-}Fl_{ox} \xrightarrow{dark} Fl_{ox} + E\text{-}Fl_{red}H_2 \quad (8)$$

Dark Reactions of Reduced Flavins with Flavoproteins. In keeping with the hypothesis detailed in the previous section, it is found that free dihydroflavins react with flavoproteins, and that the reaction rate is greater with the oxidized flavoprotein than with its semiquinone form. In most cases, the reactions are sufficiently fast that they have to be monitored with a stopped-flow apparatus. Figure 4 shows the reaction under anaerobic conditions of glucose oxidase at pH 5.3 with a tenfold excess of reduced lumiflavin 3-acetate. At this pH value the glucose oxidase semiquinone is the blue colored neutral form, which is conveniently monitored at 570 nm, where neither the oxidized nor the reduced enzyme absorbs light. The rapid production of semiquinone is observed, followed by its slower disappearance as fully reduced enzyme accumulates. The rates of both steps are directly proportional to the concentration of reduced lumiflavin acetate, allowing determination of the second-order rate constants. These are listed in Table II, together with the results obtained with reduced tetraacetylriboflavin at the same pH value. As expected from the relative rate constants for semiquinone production and full reduction, a lower concentration of glucose oxidase semiquinone is formed

TABLE II: Rates of Reaction of Flavoproteins with Free Reduced Flavins.

Flavoprotein	Conditions	k_5 ($M^{-1} s^{-1}$)	k_6 ($M^{-1} s^{-1}$)
Glucose oxidase	pH 5.3, 25 °C reduced lumiflavin 3-acetate	2.9×10^3	3.3×10^2
	pH 5.3, 25 °C reduced tetraacetylriboflavin	1.4×10^3	4.4×10^2
	pH 5.3, 3 °C reduced tetraacetylriboflavin	2.7×10^2	48
	pH 9.1, 25 °C reduced lumiflavin 3-acetate	1.5×10^2	–
D-Amino acid oxidase	pH 8.7, 25 °C reduced lumiflavin 3-acetate	1.1×10^5	80
Flavodoxin	pH 6.5, 25 °C reduced lumiflavin 3-acetate	1.0×10^3	–
	pH 7.0, 25 °C reduced lumiflavin 3-acetate	3×10^3	–
	pH 7.0, 25 °C reduced riboflavin	1.9×10^3	–
	pH 7.0, 25 °C reduced FMN	1.1×10^3	–
	pH 7.0, 25 °C reduced tetraacetylriboflavin	9.4×10^2	–

[a] The minus sign indicates that no reduction of the enzyme semiquinone was observed.

with this flavin than with reduced lumiflavin 3-acetate. The effect of temperature on reaction rates and semiquinone yield should also be noted. At pH 9.1, where the glucose oxidase semiquinone anion is formed, no reduction beyond the radical stage is observed even with considerable excess of reduced flavin. In this respect it is interesting to note that photoreduction of glucose oxidase at this pH value proceeds quantitatively to the semiquinone level (Massey and Palmer, 1966) and no further reduction is observed. This situation also applies at pH 9.1 with added flavins such as 3-methyllumiflavin and lumiflavin 3-acetate, which while enhancing considerably the photoreduction rate, do not promote photoreduction beyond the semiquinone level (results not shown).

In contrast to the relatively slow rate of reaction with glucose oxidase, reduced lumiflavin acetate reacts in the dark quite rapidly with D-amino acid oxidase to form the enzyme semiquinone anion; the subsequent reaction to give fully reduced enzyme is three orders of magnitude slower (Table II). In keeping with these results, and the hypothesis developed in the previous section, controlled photoreduction of this enzyme yields close to quantitative amounts of radical, followed by slower photoreduction to the fully reduced form (Massey and Palmer, 1966). Both steps are considerably increased in rate in the presence of catalytic amounts of 3-methyllumiflavin or lumiflavin 3-acetate (see section entitled Influence of the Competitive Inhibitor, Benzoate and Figure 5).

Table II also lists the second-order rate constants for formation of the neutral semiquinone of flavodoxin with a variety of reduced flavins. No reduction beyond the semiquinone stage was detected in these experiments, although evidence to be presented later indicates that a small equilibrium concentration of fully reduced flavodoxin is formed (see section entitled Saturation of the Photoreduction Rate). Again the results are consistent with the nearly quantitative production of the flavoprotein semiquinone in photoreduction studies (Mayhew and Massey, 1969).

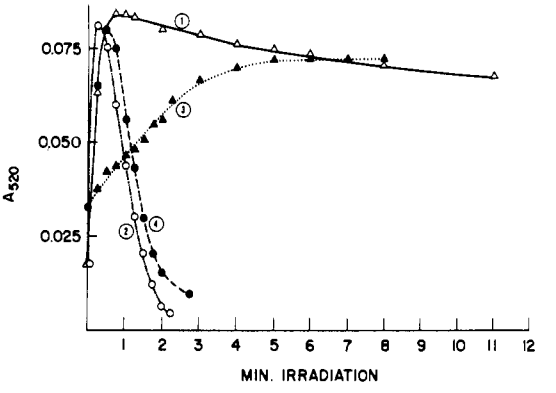

FIGURE 5: Effect of 3-methyllumiflavin and benzoate on the photoreduction of D-amino acid oxidase. Enzyme, 36.7 μM with respect to FAD content, in 0.02 M pyrophosphate, pH 8.5, containing 0.012 M EDTA, was made anaerobic and photoreduced at 19 °C as described under Materials and Methods. After each illumination period, absorbance was followed until no further changes occurred, and the spectrum was recorded. These dark changes were rapid during the phase of semiquinone accumulation, but were slow during the phase of conversion to fully reduced enzyme. The typical spectrum of the red-colored semiquinone anion was found (see Massey and Palmer, 1966). This is conveniently monitored by its increased absorbance at 520 nm, compared with that of oxidized or fully reduced enzyme. (Curve 1) No additions other than EDTA. (Curve 2) Plus 2 μM 3-methyllumiflavin. (Curve 3) Plus 100 μM benzoate. (Curve 4) Plus 2 μM 3-methyllumiflavin and 100 μM benzoate.

Influence of the Competitive Inhibitor, Benzoate, on the Photoreduction of D-Amino Acid Oxidase. Benzoate is a potent competitive inhibitor of D-amino acid oxidase (Frisell et al., 1956) and its effects on the physical properties of the enzyme have been studied in some detail (Yagi and Ozawa, 1962;

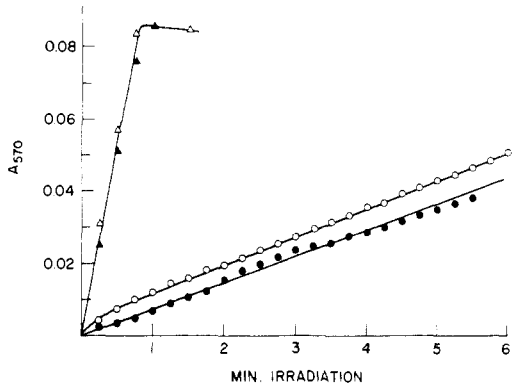

FIGURE 6: Effect of light intensity on the photoreduction of flavodoxin in the absence and presence of lumiflavin 3-acetate. Flavodoxin from *P. elsdenii*, 19–20 μM with respect to FMN content, in 0.05 M phosphate, pH 6.5, and 0.025 M EDTA, was made anaerobic and subjected to 15-s illumination periods at 25 °C as described under Materials and Methods. It was necessary to wait 5–10 min after each irradiation for dark changes to become complete. ● and ○ show the increase in A_{570} due to semiquinone production at light intensities of 3.6×10^6 ergs cm^{-2} s^{-1} and 8.4×10^6 ergs cm^{-2} s^{-1}, respectively. ▲ and △ show the results at the same light intensities, but in the presence of 2 μM lumiflavin 3-acetate.

Massey and Ganther, 1965; Antonini et al., 1966). Although possible protein conformation changes accompanying its binding (Kotaki et al., 1966) may confuse the argument, an unequivocal result of its binding should be to decrease the amount of free FAD dissociated from the holoenzyme, because of the preferential binding of benzoate to the holoenzyme:

$$\text{E} \cdot \text{FAD} \rightleftharpoons \text{E} + \text{FAD} \qquad (9)$$

$$\text{E} \cdot \text{FAD} + \text{benzoate} \rightleftharpoons \text{E} \cdot \text{FAD} \cdot \text{benzoate} \qquad (10)$$

At pH 8.5, 19 °C, the K_d for enzyme-bound flavin has been determined as 5.3×10^{-7} M (Massey et al., 1966; Yagi et al., 1975), while that for benzoate binding has been determined as 2×10^{-6} M (Quay and Massey, 1977). Because of its comparatively weak binding to the protein, appreciable concentrations of free FAD are present in equilibrium, unless the enzyme concentration is high. In terms of the hypothesis that photoreduction is mediated through free coenzyme, this would predict that D-amino acid oxidase should be one of the most readily photoreducible flavoenzymes, as previously found (Massey and Palmer, 1966). By virtue of the coupled equilibria (eq 9 and 10), it would also be predicted that photoreduction should be considerably slowed when benzoate is present. Indeed McCormick et al. (1967) state that benzoate decreases the photoreduction rate of D-amino acid oxidase, but ascribed this effect to a lowered exposure of bound FAD to solvent in the enzyme–benzoate complex.

Figure 5 shows the effect of benzoate on the photoreduction of D-amino acid oxidase in the absence and presence of catalytic amounts of 3-methyllumiflavin. In the absence of added flavin, the presence of 10^{-4} M benzoate increases the half-time of formation of enzyme–semiquinone anion from approximately 0.2 min to 1.5 min, corresponding to k_{obsd} values of 3.5 and 0.46 min^{-1}, respectively. Taking a K_d for flavin binding of 5.3×10^{-7} M (Massey et al., 1966), it can be calculated that under the conditions of curve 1 approximately 10% of the total flavin is free (4.2×10^{-6} M), corresponding to a photoreduction rate of 8.3×10^5 M^{-1} min^{-1}. In the presence of 10^{-4} M benzoate (conditions of curve 2), the calculated free FAD concentration is 7.8×10^{-7} M, corresponding to a photoreduction rate of 5.9×10^5 M^{-1} min^{-1}. When catalytic amounts of lumiflavin 3-acetate (results not shown) or 3-methyllumiflavin are added, complete semiquinone formation is obtained within the first 15 s of irradiation period, and complete reduction obtained with approximately 2-min irradiation (curve 2). As expected, benzoate has very little effect on the catalysis caused by the model flavin (curve 4), which does not bind to the enzyme.

Saturation of the Photoreduction Rate with Light Intensity. It is evident that the catalytic effect of model flavins on photoreduction of flavoproteins is explained satisfactorily by the reaction sequence 4–6, 2. The intriguing possibility that the same basic reactions occur in the uncatalyzed reaction was further tested by examining the effect of light intensity on the photoreduction rate. Free flavins are completely reduced under the conditions employed in this work within a few seconds of irradiation. If the comparative slowness of the photoreduction of flavoproteins were due to the slow reaction of free reduced coenzyme in a subsequent dark reaction with the holoenzyme, then it would be expected that, over a certain threshold level, the rate of photoreduction would be independent of light intensity. This prediction has been verified experimentally with two flavoproteins, glucose oxidase (results not shown) and flavodoxin (Figure 6). This figure shows the accumulation of the blue neutral semiquinone by 15-s irradiation periods followed by dark equilibration. The four experiments were performed in the absence and presence of catalytic concentrations of lumiflavin 3-acetate, using light intensities differing by a factor of greater than two. It can be seen that, with both the uncatalyzed and catalyzed reactions, the rate of semiquinone accumulation is independent of the light intensity. At both light intensities the free flavin was completely reduced in less than 5 s, as judged visually by loss of the typical oxidized flavin fluorescence. That the photoreduction in the absence of added flavin is due to equilibrium amounts of free FMN is also confirmed by fluorescence observations. The flavoprotein has weak fluorescence, approximately 0.5% that of an equivalent concentration of FMN. Immediately after each 15-s irradiation period this fluorescence is quenched, but reappears over a 5-min period. The yield of semiquinone obtained after each 15-s illumination in the two lower curves of Figure 6 is approximately 1% of the total possible, consistent with 0.5% free FMN in equilibrium with apoprotein and holoenzyme (eq 7) and the reactions of eq 4–6, 2.

The presence of 0.5% free FMN under the conditions of Figure 6 implies a K_d for FMN dissociation from the flavodoxin holoprotein of $\sim 5 \times 10^{-10}$ M. This value is very close to that reported previously (4.3×10^{-10} M) at the same pH value (Mayhew, 1971).

In the case of the added lumiflavin acetate the production of flavodoxin semiquinone from reduced lumiflavin acetate is very easy to follow. During the illumination period semiquinone is produced according to eq 4–6, 2, and the added flavin is maintained at a steady-state level which is largely in the reduced state, as judged by the weak fluorescence observable immediately after the light is switched off. In the following dark period, the formation of approximately 4×10^{-6} M flavodoxin radical (i.e., twice the concentration of added lumiflavin acetate) can be measured by the increase in absorbance at 570 nm; during this time the strong fluorescence of oxidized lumiflavin acetate returns. This dark reaction takes approximately 5–10 min for completion, with a half-time of 1–2 min. After maximum semiquinone is produced there is a small extent of formation of fully reduced flavodoxin. The extent of full reduction finally reached appears to be due to a redox equilibrium and is dependent on the concentration of lumiflavin acetate added. It should be noted that there is a considerable thermodynamic barrier to reduction of flavodoxin beyond the semiquinoid level. The E_0' pH 7 value of the couple flavodoxin

semiquinone/reduced flavodoxin is −0.371 V (Mayhew et al., 1969) while that for the $Fl_{ox}/Fl_{red}H_2$ couple with lumiflavin acetate is −0.246 V (Müller and Massey, 1969).

The possibility was also checked of complications arising from photocatalysis of the comproportionation reaction (eq 3). Flavodoxin is somewhat unusual among flavoproteins in exhibiting this reaction at a reasonable rate (Mayhew and Massey, 1973). Accordingly reduced flavodoxin was prepared by anaerobic titration with dithionite, an equal amount of oxidized flavodoxin added from a side arm, and the formation of flavodoxin semiquinone monitored at 570 nm. Under the same conditions as those of Figure 6 the formation of semiquinone was a slow second-order process, with a rate constant of 2×10^4 M^{-1} min^{-1}. This comproportionation reaction was not influenced by several periods of illumination.

General Discussion

From the results presented it is clear that trace amounts of free flavins will catalyze the photoreduction of flavoproteins, via the reaction sequence of eq 4–6, 2. All flavins tested act as catalysts, including the natural coenzymes. In addition to the ones already described, we have determined that similar stimulations in the photoreduction rate occur with the following flavoproteins: lipoyl dehydrogenase, L-amino acid oxidase, lactate monooxygenase, melilotate hydroxylase, *p*-hydroxybenzoate hydroxylase, ferredoxin-$NADP^+$ reductase, Old Yellow Enzyme, and milk xanthine oxidase. Thus we have every reason to believe that this is a general phenomenon and provides a useful method of generating readily not only the semiquinones but in many cases also the fully reduced flavoproteins for mechanistic studies.

Although this aspect has not yet been explored in detail, the results presented in Table II suggest further applications of the method. With glucose oxidase the ratio of the rates of the first and second reduction steps (eq 5 and 6) is seen to differ depending on the flavin employed, as well as on the temperature and the pH. As this ratio should determine the yield of semiquinone, it should be possible to vary this yield in photochemical reactions by variation of these parameters. In this respect it should be noted that several flavoproteins give low yields of semiquinone on photochemical reduction. Some examples are lactate monooxygenase, Old Yellow Enzyme, and *p*-hydroxybenzoate hydroxylase. With another flavoprotein monooxygenase, melilotate hydroxylase, no semiquinone has been detected on photoreduction (Strickland and Massey, 1973). This enzyme is presumably one in which $k_6 > k_5$, in contrast to the more general situation, where $k_5 > k_6$. Alternatively it might be an example where thermodynamic radical stabilization is lacking, leaving $2e^-$ transfer as the preferred mechanism of reduction.

The probability exists that, even in the absence of added flavin, the mechanistic route of the photochemical reduction may be the same, with the reaction being promoted by the small equilibrium concentration of free natural flavin coenzyme present. This may not be the only route available, but the results presented argue strongly for its providing a major contribution to the photoreduction process. We hope to test this concept further by studying photoreduction of enzymes in which the flavin is covalently linked to the protein.

Finally some comment is appropriate concerning comparisons of photoreduction rates among different flavoenzymes. It has been our observation that long term storage of some enzymes results in rather subtle changes in properties. For example, glucose oxidase, and ferredoxin-$NADP^+$ reductase when freshly prepared, are practically devoid of fluorescence. However, on aging (even at −20 °C), fluorescence develops. Such aged enzyme, if used untreated, is much more rapidly photoreduced than freshly prepared enzyme. This is presumably due to some hydrolysis of the flavin coenzyme, which then promotes catalysis of the photoreduction, since much of the effect can be removed by dialysis or gel filtration. However, even after this treatment, faster photoreduction rates are observed than with freshly prepared enzyme. We have not investigated the cause of this phenomenon; it could be due to partial modification of the protein so that the binding strength of the flavin association was somewhat weaker.

In conclusion we wish to emphasize that there are at least two different types of photochemical reactions of flavoproteins. In cases where photoaddition reactions have been observed (De Kok and Veeger, 1967; Ghisla and Massey, 1975) the photochemical event involves the excited state of the flavoprotein interacting with the photosubstrate. In the present case of flavoprotein reduction with EDTA as photosubstrate, the reaction does not involve an excited state of the holoprotein, nor direct contact of EDTA with the enzyme. Instead, it appears to be due to an interaction between free flavin and enzyme bound flavin. Depending on the apoprotein, which may or may not stabilize the flavin radical state, this "interflavin" oxidoreduction could involve one $2e^-$- or two $1e^-$-transfer steps (Hemmerich, 1977). The demonstration of facile interaction of a wide variety of flavoproteins with extraneous flavin lends support for the concept that, in normal catalysis of flavoenzymes containing more than one flavin prosthetic group, similar interflavin electron transfer may occur.

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