Photoreduction of Flavoproteins and Other Biological Compounds Catalyzed by Deazaflavins†

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ABSTRACT: Deazaflavins have been found to act as potent catalysts in the photoreduction of flavoproteins in the presence of EDTA and other "photosubstrates". In distinction to the catalysis brought about by normal flavins which involves dark reaction of the photoreduced flavin catalyst, the mechanism of the catalysis by deazaflavins has been shown to involve unstable, strongly reducing radicals which are generated by photolysis of a preformed covalent dimer. By this new method it is possible to reduce not only flavoproteins but a variety of other redox proteins, including heme proteins and iron-sulfur proteins. By virtue of its great catalytic efficiency, it is possible to employ concentrations of deazaflavin sufficiently low as not to interfere with the spectral evaluation of the reduced proteins obtained.

In the preceding publication (Massey et al., 1978), we demonstrated that free flavins were very efficient catalysts in the photoreduction of a wide variety of flavoproteins. It was established that the catalytic effect was due to photoreduction of the flavin followed by dark reactions between the free dihydroflavin and the flavoproteins which may occur either by $1e^-$ or $2e^-$ transfer. We also tested deazaflavins as possible catalysts in such photoreductions and have found them to be even more efficient catalysts, especially in the capacity to generate readily the 1,5-dihydro forms of flavoproteins by strict single electron transfers. Thus, it was found that the mechanism of their catalytic effect is completely different from that of normal flavins and, as detailed in this paper, appears to involve the photochemical generation of the deazaflavin radical, which combines high reactivity with a very low redox potential. Because of this property it is possible with catalytic quantities of deazaflavins to reduce photochemically not only flavoproteins, but a variety of other compounds including heme proteins and iron-sulfur proteins. We believe that this simple method may have wide applications in biological problems by providing a catalytic source of electron equivalents of very low potential. An abbreviated account of this work has been reported elsewhere (Massey and Hemmerich, 1977).

Materials and Methods

Photochemical studies were carried out as described in the preceding paper (Massey et al., 1978), except that, where appropriate, deazaflavin was substituted for flavin. Ethylenediaminetetraacetate (EDTA)[1] was used as the ultimate source of reducing equivalents in most of the photochemical reactions; however, other sources such as simple amino acids, nitrogenous buffers such as tris(hydroxymethyl)aminomethane and oxalate can also be used.

Deazaflavins. Two deazaflavins were employed interchangeably. The deazaflavins were prepared by a new synthetic route (Janda and Hemmerich, 1976) as were the 1,5-dihydro derivatives (M. Janda and P. Hemmerich, unpublished). In addition to deazariboflavin ($dRFl_{ox}$ and $dRFl_{red}H_2$, respectively), a simplified type of deazaflavin was employed which lacks the 7,8-dimethyl substituents and bears a methyl instead of the ribityl group: 3,10-dimethyl-5-deazaisoalloxazine. This deazaflavin is abbreviated as dFl_{ox} and $dFl_{red}H_2$ to distinguish it from deazariboflavin. No significant differences were detected in the speed or efficiency of photoreduction when dFl_{ox} was used instead of $dRFl_{ox}$.

Enzymes. The flavoproteins used were obtained as described in the preceding paper (Massey et al., 1978). Chloroperoxidase was a gift from Dr. L. Hager, cytochrome *c* peroxidase was a gift from Dr. T. Yonetani. Cytochrome *c* (Grade V) and NAD^+ were obtained from The Sigma Chemical Co.; catalase (crystalline) was obtained from Calbiochem. Deflavoxanthine oxidase was prepared by the method of Komai et al. (1969).

Results and Discussion

Photochemical Reduction of Flavodoxin. In contrast to the photoreduction catalyzed by free flavins, where conversion of flavodoxin to the semiquinoid state occurs readily but further reduction beyond this state is very difficult (Massey et al., 1978), flavodoxin is readily photoreduced to the fully reduced state in the presence of deazaflavins. This effect is illustrated in Figure 1. With short periods of illumination, the blue-colored neutral radical accumulates almost quantitatively, but on further illumination is converted rapidly to the fully reduced level. While a comparatively large concentration of deazariboflavin was employed in this experiment, similar effects, but occurring more slowly, are observed with molar ratios of $dRFl_{ox}$ to flavodoxin of 1/100. A valuable clue to the mechanism of photoreduction was obtained in such experiments. The oxidized flavoprotein spectrum shown in Figure 1 was recorded after photoreaction of the $dRFl_{ox}$ with EDTA in the side arm of the anaerobic cuvette and then mixing in the dark with the flavodoxin. Even after several hours in the dark there was no reduction of the flavodoxin. With similar experiments employing normal flavins, flavodoxin semiquinone is formed

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[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; UV, ultraviolet; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; $dRFl_{ox}$, $dRFlH^\circ$, and $dRFl_{red}H_2$, the oxidized, radical and fully reduced forms of 5-deazariboflavin; dFl_{ox}, $dFlH^\circ$, and $dFl_{red}H_2$, the corresponding forms of 3,10-dimethyl-5-deazaisoalloxazine; $(dFlH)_2$, the covalent radical dimer of either of the above deazaflavins; Fl_{ox}, FlH°, and $Fl_{red}H_2$, oxidized, radical, and fully reduced forms of free flavins. The prefix E with any of the above is used with the appropriate enzyme-bound form.

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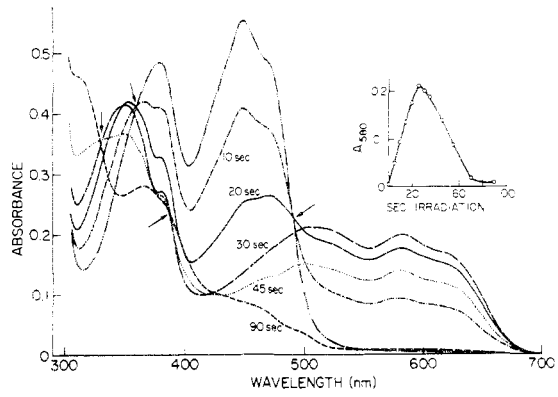

FIGURE 1: Effect of $dRFl_{ox}$ on the photoreduction of flavodoxin. Conditions: 53.3 μM flavodoxin, 6.6 μM $dRFl_{ox}$, 15 mM EDTA, 20 mM pyrophosphate, pH 8.5, 25 °C. The EDTA and $dRFl_{ox}$ were illuminated in the side arm of the anaerobic cuvette for 2 min before mixing with the flavodoxin. No spectral changes occurred even over several hours in the dark (— · · · —). Short periods of illumination were then given using a 250-W slide projector lamp at the low intensity setting (~2×10^6 ergs cm^{-2} s^{-1}). The inset shows the A_{580} values obtained after each illumination. Isosbestic points at 490 and 358 nm were observed up to 20-s irradiation and were replaced by a new set at 387 nm and 329 nm at longer irradiation times.

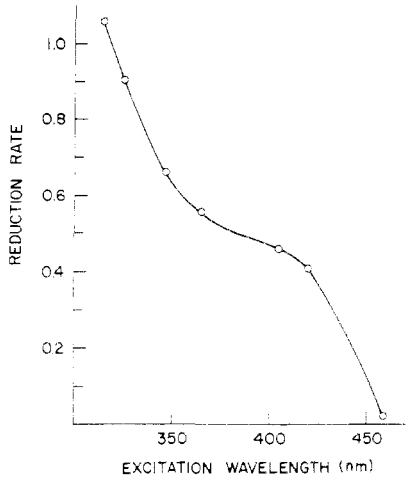

FIGURE 2: Action spectrum of the photoreduction of flavodoxin. Deazariboflavin was illuminated in the side arm of an anaerobic cuvette with EDTA until the intense blue fluorescence of $dRFl_{ox}$ disappeared. The contents of the side arm were then mixed with flavodoxin, and the cell was illuminated in individual experiments through a set of narrow band pass filters (Balzers, Liechtenstein). Spectra were recorded after each illumination period, and the rate of photoreduction to the semiquinone level was determined from plots of the kind shown in the inset of Figure 1. The light intensity transmitted by each filter was determined with a Kipp and Zonen type CA 1 bolometer and ranged from 2.12×10^3 ergs cm^{-2} s^{-1}, with the 315-nm filter to 3.08×10^4 ergs cm^{-2} s^{-1}, with the 459-nm filter. The observed photoreduction rates were then corrected to the same light intensity.

within a few minutes in the stoichiometry of 2 equiv of flavodoxin semiquinone per equiv of photoreduced flavin (Massey et al., 1978). The production of semiquinone and its conversion to fully reduced protein in Figure 1 are thus absolutely dependent on subsequent illumination of the mixture.

The action spectrum for photoreduction of flavodoxin was determined in experiments such as those of Figure 1, but employing a slightly greater than equimolar concentration of deazaflavin. The latter was illuminated in the side arm with EDTA and then mixed with the flavodoxin solution. As described above, no dark reduction was ever observed, even with such high concentrations of deazaflavin. The solutions, in

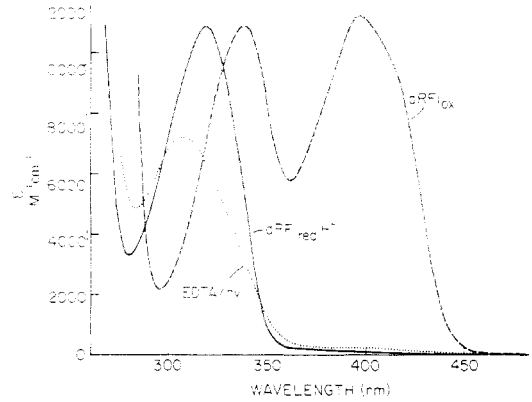

FIGURE 3: Spectra of $dRFl_{ox}$, $dRFl_{red}H^-$, and the product of photoreduction of $dRFl_{ox}$ in the presence of EDTA. A solution of $dRFl_{ox}$ in 0.1 M pyrophosphate, pH 8.6, was reacted anaerobically with a trace of $NaBH_4$ to yield the spectrum of $dRFl_{red}H^-$, or illuminated in the presence of 15 mM EDTA for 30 s with a light intensity of ~8×10^6 ergs cm^{-2} s^{-1}. Extinction coefficients were calculated on the basis of 12000 M^{-1} cm^{-1} for the starting $dRFl_{ox}$ (Spencer et al., 1976).

separate experiments, were then illuminated through a series of narrow band pass filters. In each case the tent-shaped reaction profile shown in the inset of Figure 1 was obtained; the rate of semiquinone production was obtained from such plots, and then corrected to the same light intensity. The results of such a series of experiments are shown in Figure 2. Essentially no photoreduction was observed at wavelengths higher than 460 nm; the maximal rate observed was with the lowest wavelength narrow band pass filter available to us. As will be discussed in a later section (General Discussion) the photochemical action spectrum appears to have components both of $dRFl_{ox}$ and the product of illumination of $dRFl_{ox}$ and EDTA.

The flavodoxin used in the above studies was that obtained from *P. elsdenii*. Qualitatively the same results (i.e., reduction to the semiquinonoid state followed by full reduction) have also been obtained with the flavodoxins from *Azotobacter vinelandii* and *Desulfovibrio vulgaris* (Scherings et al., 1977).

Nonidentity of the Photoproduct of Deazariboflavin and EDTA with 1,5-Dihydrodeazariboflavin. When riboflavin or any normal flavin analogue is subjected to visible light in the presence of EDTA, the product is the corresponding 1,5-dihydroflavin (Frisell et al., 1959). However, the photoproduct with deazaflavin is distinctly different from that of authentic 1,5-dihydrodeazaflavin, in many of its properties—spectroscopic, fluorescence, and chemical reactivity. Figure 3 illustrates the differences in absorption spectrum. As detailed previously (Spencer et al., 1976) deazariboflavin is readily reduced to 1,5-dihydrodeazariboflavin by borohydride and is distinguished by a single-banded near-UV spectrum with λ_{max} = 320 nm and extinction coefficient nearly identical with that of the oxidized form. The dihydro form has a pK of 7.0–7.2 (Spencer et al., 1976, Stankovich and Massey, 1976) due to ionization of the N(1) proton; hence the spectrum of the borohydride-reduced material shown in Figure 3 is that of the anion, $dRFl_{red}H^-$. The spectrum of the neutral species, $dRFl_{red}H_2$, is, however, only marginally different in this wavelength region (Spencer et al., 1976; Stankovich and Massey, 1976). In contrast, the spectrum of the EDTA–light product is quite different, with a wavelength maximum at 307 nm, and an ϵ_{307} value of 7500 M^{-1} cm^{-1}, based on the ϵ_{400} value of 12 000 M^{-1} cm^{-1} for the starting $dRFl_{ox}$. Like $dRFl_{red}H_2$, the position of this band also varies with pH, with an apparent pK of ~7.5, but the extinction coefficient does not

TABLE I: Chemical Reactivities of Dihydrodeazariboflavin and the Products of the EDTA-Light Reaction.[a]

Compound	Second-order rate constants (M^{-1} s^{-1}) with $K_3Fe(CN)_6$	Cytochrome *c*	Lumiflavin 3-acetate
$dRFl_{red}H^-$	1.07	Very small ($t_{1/2}$ h)	1.67×10^2
$dRFl_{ox}$/EDTA/light	3×10^3 (73%) 6×10^2 (27%)	1.4×10^2	2.7 (70%) 27 (30%)

[a] The reactions were carried out in 0.05 M pyrophosphate, pH 8.6, 25 °C. The products of the EDTA-light reaction were also obtained by illumination under these conditions (see Figures 3 and 4 for details). The reaction rates with lumiflavin 3-acetate were obtained with a Cary 17 recording spectrophotometer at 390 nm, using a wide range of lumiflavin acetate concentrations, including ones well below that of the reduced deazaflavin species. The observed reoxidations were accurately first order and the observed first rate constants directly proportional to flavin concentration. This is presumably due to the flavin acting as a catalyst ($dFl_{red}H^- + Fl_{ox} \rightarrow dFl_{ox} + Fl_{red}H^-$; $Fl_{red}H^- + H^+ + O_2 \rightarrow Fl_{ox} + H_2O_2$). The reactions with ferricyanide were carried out with a stopped-flow apparatus, under pseudo-first-order conditions, using $Fe(CN)_6^{3-}$ in excess, and monitoring dFl_{ox} formation at 390 nm. The reaction with $dRFlH^-$ was accurately first order, with k_{obsd} proportional to $[Fe(CN)_6^{3-}]$. The first-order plots for dFl_{ox} formation from the dFl_{ox}/EDTA/light reaction product were biphasic. These were analyzed by the method given by Frost and Pearson (1961) for two independent reactions to yield the rate constants and proportions (shown in brackets). The reaction with cytochrome *c* was followed at 550 nm, also under pseudo-first-order conditions, but with the reduced deazaflavin species in excess. Although it was clear that the reaction mixture was not homogeneous, no accurate estimation of proportions of the two reactants was possible because of the practical necessity of using an excess of the reduced deazaflavin species.

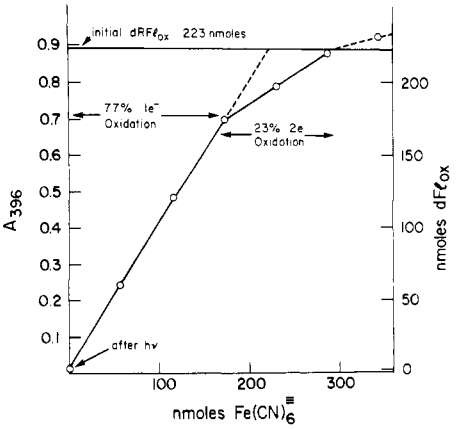

FIGURE 4: Determination of the oxidation state of the photoproduct of $dRFl_{ox}$ and EDTA. Deazariboflavin, 223 nmol, in 56 mM pyrophosphate, pH 8.6, and 10 mM EDTA was irradiated with visible light for 30 s at an intensity of $\sim 8 \times 10^6$ ergs cm^{-2} s^{-1} under anaerobic conditions, 25 °C (cf. Figure 3). Four equal aliquots of $K_3Fe(CN)_6$ were added sequentially from separate side arms of the anaerobic assembly. After each addition from the first three side arms there was a rapid formation of $dRFl_{ox}$, as judged by the change in the absorption spectrum. The changes in A_{396}, the absorption maximum of $dRFl_{ox}$, are shown here. On the fourth addition of $K_3Fe(CN)_6$, the formation of $dRFl_{ox}$ was very slow, requiring approximately 2 h for completion. Air was then admitted, and the titration with $K_3Fe(CN)_6$ was continued. After the fifth addition the only spectral changes observed were those due to the added $K_3Fe(CN)_6$.

vary significantly with pH. These results are at variance with those reported by Edmondson et al. (1972) who show a much higher extinction for the EDTA-light product. We have no explanation for the discrepancy, but have observed consistently in many experiments the results shown in Figure 3. The fluorescence properties of the EDTA-light product are also distinctly different from those of $dRFl_{red}H^-$. While both have emission maxima centered around 400 nm, $dRFl_{red}H^-$ has excitation maxima at 255 and 316 nm (Stankovich and Massey, 1976), while the EDTA-light product has excitation maxima at 258 and 304 nm. Furthermore, its fluorescence is less intense, being only 0.29 that of $dRFl_{red}H^-$.

In addition to these differences in physical properties, the two species also differ profoundly in chemical reactivity. The EDTA-light product reacts very rapidly (in the dark) with ferricyanide and comparatively rapidly with cytochrome *c*; on the other hand it reacts rather slowly with free flavins such as riboflavin or lumiflavin 3-acetate, and not at all with flavodoxin. In contrast, dihydrodeazariboflavin reacts extremely slowly with ferricyanide and cytochrome *c* (this work), moderately rapidly with free flavins (Spencer et al., 1976; this work) and does react anaerobically with flavodoxin, albeit slowly. Both species react very slowly with O_2, with $t_{1/2}$ values in the order of days. All of these reactions, with both compounds, are very much accelerated in the light. Table I gives rate constants for several of these reactions, carried out under identical conditions (pH 8.6, 25 °C). The two entries given for the product of the $dRFl_{ox}$-EDTA light reaction are because the photochemical reaction leads to two products, which can be differentiated in the stopped-flow studies on the basis of their kinetics. Such analysis also permits the determination of their proportions, shown in brackets in the table. Entirely similar results were obtained with the product from EDTA and 3,10-dimethyl-5-deazaisoalloxazine.

Evidence for the Dimeric Nature of the Final Product of the Light Reaction. A clue to the chemical nature of the product of the EDTA-light reaction came from its rapid reaction with $Fe(CN)_6^{3-}$. Titration experiments with $dRFl_{red}H^-$ showed that, as expected, 2 equiv of $Fe(CN)_6^{3-}$ were required for the formation of $dRFl_{ox}$. If the product from the light reaction were a covalent derivative of some EDTA moiety and 1,5-dihydrodeazaflavin, the same stoichiometry would be expected on theoretical grounds. However, from experiments such as those shown in Figure 4, it is clear that the major product of the EDTA-light reaction consumes 1 equiv of $Fe(CN)_6^{3-}$ per equiv of $dRFl_{ox}$ produced. This reaction occurs very rapidly. The remaining product (23% in the experiment of Figure 4) reacts very slowly, over a period of hours, to produce $dRFl_{ox}$, but consuming 2 equiv of $Fe(CN)_6^{3-}$ in the process. The one-electron equivalence of the major product implies that it is formally at the deazaflavin radical state of reduction. This would be most logically represented by a dimeric structure, in keeping with the observed lack of an EPR signal. The possible structure of the dimer, and a plausible route for its formation, are given in Figure 5. Thus the first (and slower) step is envisaged as a "group-transfer" reaction, with the adduct formed undergoing a secondary (and faster) reaction with dFl_{ox}*, resulting in dimer formation and liberation of the oxidized substrate residue. That such dimers are indeed formed has been shown by their isolation from photochemical reduction of 3,10-dimethyl-5-deazaflavin in the presence of oxalate, and NMR characterization (see Appendix). Such dimers have similar chemical reactivities to the products of the $dRFl_{ox}$-EDTA photoreaction shown in Table I (see Appendix).

Photochemical Generation of Dimer from Mixtures of $dRFl_{ox}$ and $dRFl_{red}H_2$. A product with properties very similar to those described in the previous section is also obtained anaerobically on illumination of equimolar mixtures of $dRFl_{ox}$

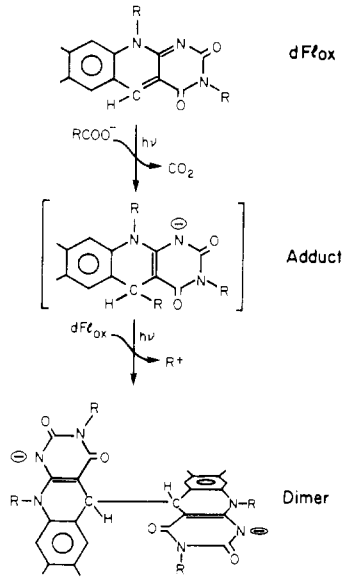

FIGURE 5: Hypothetical scheme for the production of deazaflavin dimer in the photochemical reaction. The intermediate adduct is envisaged as being even more photochemically reactive than the starting dFl_{ox}.

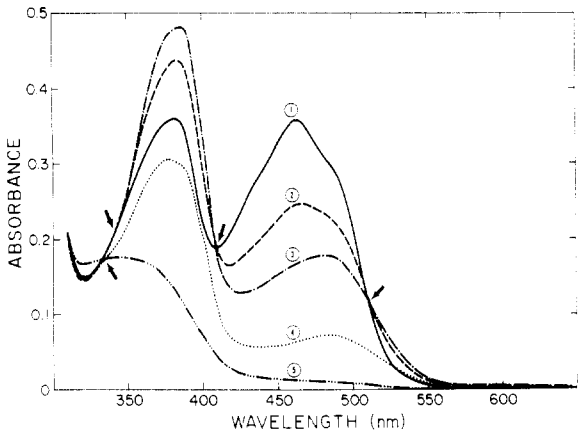

FIGURE 6: Effect of $dRFl_{ox}$ on the photoreduction of Old Yellow Enzyme. Conditions, 34 μM Old Yellow Enzyme, 100 mM pyrophosphate, 15 mM EDTA, and 1.7 μM $dRFl_{ox}$, under anaerobic conditions, 25 °C. Illumination was carried out at an intensity of $\sim 2 \times 10^6$ ergs cm^{-2} s^{-1} and spectra were recorded after each illumination period. Selected spectra only are shown. (Curves 1–5) After zero, 15 s, 25 s, 1.25 min, and 11 min of illumination.

and $dRFl_{red}H_2$, in the absence of photosubstrates such as EDTA or oxalate. This photoreaction is very rapid, similar to that described in Figure 3 for the EDTA–light reaction. The possibility of forming mixed dimers is very real since the reaction proceeds equally well with mixtures of $dRFl_{ox}$ and $dFl_{red}H_2$ and with dFl_{ox} and $dRFl_{red}H_2$. In all cases the photoproduct is stable to O_2 in the dark but is rapidly converted to the corresponding oxidized deazaflavin(s) on subsequent illumination with visible light.

Photochemical Reduction of Other Flavoproteins. We have tested the catalytic effects of deazaflavins on the photoreduction of a variety of flavoproteins, including the following: glucose oxidase, D-amino acid oxidase, L-amino acid oxidase, Old Yellow Enzyme, ferredoxin-$NADP^+$ reductase, melilotate hydroxylase, *p*-hydrobenzoate hydroxylase, lactate monooxygenase, egg white binding protein complexed with riboflavin, and xanthine oxidase. In all cases catalytic concentrations of deazaflavin resulted in substantial increases in the rate of photoreduction, whether the flavoprotein employed was one

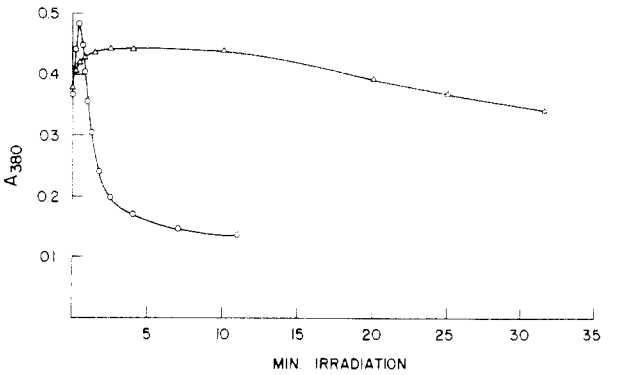

FIGURE 7: Catalytic effect of $dRFl_{ox}$ on the formation of the anion semiquinone of Old Yellow Enzyme and the subsequent conversion to fully reduced enzyme. Conditions, as in Figure 6 ± $dRFl_{ox}$. (Δ) No additions; (O) plus 1.7 μM $dRFl_{ox}$.

in which the blue neutral semiquinone or one in which the red anionic semiquinone is stabilized.

The results with Old Yellow Enzyme are shown in Figures 6 and 7. Figure 6 shows selected spectra recorded at various irradiation times, to illustrate the facile and nearly quantitative formation of the red-colored anionic semiquinone with short periods of illumination. That predominantly the semiquinone is produced under these conditions is indicated by the isosbestic points at 342, 409, and 511 nm. On longer illumination these isosbestic points are lost, to be replaced with a single isosbestic point at 335 nm, showing the conversion of semiquinone to fully reduced enzyme in this phase. Figure 7 illustrates the dramatic catalytic effect of deazaflavin on both phases of the reduction.

With the exception of glucose oxidase at high pH values, and lactate monooxygenase, catalysis of photoreduction of the flavoproteins to the fully reduced state was observed. At pH 9.1 deazaflavin catalyzes effectively the photoreduction of glucose oxidase to its anion radical form but does not promote reduction beyond that stage. At pH 5.3, where the neutral semiquinone is formed, deazaflavin catalyzes the photoreduction both to the semiquinone and fully reduced forms.

With lactate monooxygenase, deazaflavins catalyze effectively photoreduction to the red anionic semiquinone form. If EDTA is used as the ultimate electron donor in the photoreaction, some fully reduced enzyme can be produced. However, this appears to be due to glyoxylate, a breakdown product from EDTA (Enns and Burgess, 1966), which is a substrate for lactate monooxygenase (Massey and Ghisla, 1975). When glycine is used instead of EDTA in the photoreaction, deazaflavins catalyze effectively the production of semiquinone, but no reduction beyond that state is observed.

With the flavoprotein hydroxylases, *p*-hydroxybenzoate hydroxylase, and melilotate hydroxylase, little or no semiquinone accumulates and the enzymes are photoreduced easily to the fully reduced state (Spector and Massey, 1972; Strickland and Massey, 1973). In the presence of deazaflavins the photoreduction is accelerated as with other flavoproteins, but no increase in semiquinone yield is obtained.

With lipoyl dehydrogenase, the fully reduced enzyme is also readily obtained in the presence of deazaflavins. This enzyme is reduced by its substrates, NADH or dihydrolipoyl compounds, to a red two-electron reduced state (Massey et al., 1960) which appears to be due to an internal charge-transfer complex between an active site thiolate anion (formed by reduction of the active site disulfide) and the enzyme-bound FAD in its oxidized form (Abramovitz and Massey, 1976). The

enzyme can also exist in a four-electron reduced form, in which both the FAD and active center disulfide are reduced (Massey and Veeger, 1961). Photoreduction with EDTA results in the slow conversion to the two-electron reduced form; reduction beyond this stage is achieved with difficulty (Massey and Palmer, 1966). In the presence of deazaflavins photoreduction to the four-electron reduction stage occurs readily, with intermediate accumulation of the two-electron reduced form. However, this process appears to be complex. Short bursts of illumination appear to produce transient enzyme flavin radicals, which then relax rapidly to give the spectrum of the normal two-electron reduced form. Thus immediately after illumination in the presence of deazaflavin there is much more absorbance at 600 nm than that due to the charge-transfer complex, but this decays to the value typical of the charge-transfer complex. The rapidity of this reaction ($t_{1/2} \sim 1$ min) has precluded our obtaining accurately the initial spectrum. Flash photolysis experiments will probably be required to investigate this phenomenon in detail.

With the other flavoproteins studied, results comparable to those illustrated in Figures 1 and 6 were obtained. In keeping with previous studies (Massey and Palmer, 1966; Massey et al., 1978) the semiquinones formed with ferredoxin-$NADP^+$ reductase and the egg-white binding protein were the blue-colored neutral species, while D-amino acid oxidase and L-amino acid oxidase yielded the red-colored semiquinone anions. Another enzyme which yields neutral radical intermediates is NADPH-cytochrome P-450 reductase. This interesting enzyme has been shown to contain equimolar amounts of FAD and FMN as prosthetic groups and exhibits a complex pattern of spectral changes on anaerobic titration with dithionite (Iyanagi et al., 1974; Vermilion and Coon, 1977). The various oxidation states of the enzyme can be represented; $F_1 \cdot F_2$ (oxidized), $F_1H^\circ \cdot F_2$ (one electron reduction), $F_1H^\circ \cdot F_2H^\circ$ and $F_1H_2 \cdot F_2$ (two-electron reduction), $F_1H_2 \cdot F_2H^\circ$ (three-electron reduction), and $F_1H_2 \cdot F_2H_2$ (four-electron reduction). All four reduction states, with spectra very similar to those found on dithionite reduction, are readily produced by photoreduction with EDTA in the presence of deazaflavin. These results will be published in full elsewhere (J. Vermilion, V. Massey, and M. J. Coon, to be published).

Photoreduction of NAD^+ to NAD Radical Dimer. When an anaerobic solution of NAD^+ at pH 8.5 is illuminated in the presence of EDTA and a catalytic amount of deazaflavin, a new species is produced slowly with an absorption maximum at 338 nm. The product appears to be NAD radical dimer since, like the latter, it is nonfluorescent (Burnett and Underwood, 1968; Land and Swallow, 1968) and fails to exhibit any coenzyme activity with pyruvate and rabbit muscle lactate dehydrogenase (Stein and Swallow, 1958). The dimeric nature of the product is further indicated by its reconversion to enzymatically active NAD^+ by $K_3Fe(CN)_6$; 1 equiv of $K_3Fe(CN)_6$ per NAD^+ formed is consumed in this reaction. Hence, the immediate reaction product of the photochemical reaction with NAD^+ must be NAD radical, which is known to react very rapidly with itself to form dimer (Land and Swallow, 1968).

The rapid oxidation of the dimer to NAD^+ by ferricyanide and the subsequent reduction of NAD^+ by yeast alcohol dehydrogenase plus ethanol offer two independent methods for the determination of the extinction coefficients of the dimer. By ferricyanide titration the ϵ_{259} and ϵ_{338} values of the dimer were estimated as 33 900 M^{-1} cm^{-1} and 6600 M^{-1} cm^{-1}, respectively. From the yield of NADH obtained after the addition of ethanol and alcohol dehydrogenase the corresponding values were 34 700 and 6750 M^{-1} cm^{-1}. Both with respect to

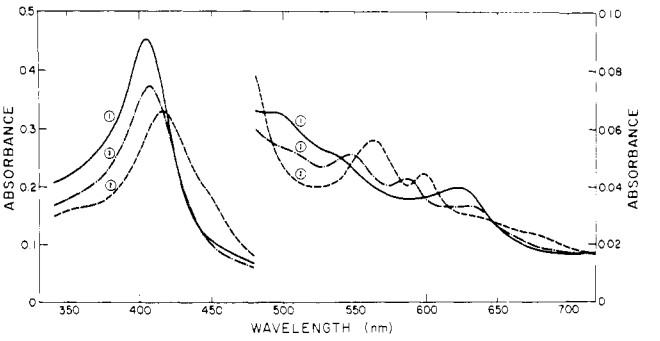

FIGURE 8: Reduction of catalase photochemically in the presence of EDTA and dFl_{ox}. Conditions: catalase, ~3.8 μM, in 20 mM pyrophosphate, pH 8.7, containing 10 mM EDTA and 1.8 μM dFl_{ox}, 25 °C. (Curve 1) After anaerobiosis, before light. (Curve 2) After 100-min irradiation at an intensity of $\sim 8 \times 10^6$ ergs cm^{-2} s^{-1}. Essentially the same spectrum was achieved with 50-min irradiation. (Curve 3) Immediately after admitting air.

wavelength maxima and extinction coefficients, the photochemically produced dimer thus has properties virtually identical with those reported by Burnett and Underwood (1968) for the dimer prepared electrolytically (λ_{max} 259 nm, ϵ 33 100; 340 nm, ϵ 6650).

The formation of NAD dimer is strong presumptive evidence for the photoreductive effect of deazaflavin being due to production of deazaflavin radicals $dFlH^\circ$ as the active reducing species, arising from photodissociation of the dimer $(dFlH)_2$. This point will be considered further in the General Discussion section.

Photoreduction of Heme Proteins with Deazaflavins as Catalyst. The following heme proteins have been shown to be reduced readily by illumination under anaerobic conditions with EDTA and catalytic amounts of deazaflavin: cytochrome *c*, chloroperoxidase, cytochrome *c* peroxidase (this work, results not shown), and cytochrome P-450 (Peterson et al., 1977). As a tribute to the reductive power of this system, it was also found that catalase could be reduced, albeit slowly. The results of one such experiment are shown in Figure 8. On illumination the spectrum of the oxidized enzyme with absorption maxima at 622 and 404 nm changed to a form with absorption maxima at 579, 562, and 415 nm. On admitting air, the starting spectrum was almost completely regained within a few minutes, with the transient appearance of an intermediate, with absorption maxima at 586 and 546 nm, shown in Figure 8. Catalytic assays performed after reoxidation showed that the enzyme had lost less than 10% of its activity as a result of the rather prolonged light irradiation. It should be noted that catalase is not converted to the Fe^{2+} form by reaction with a considerable excess of $Na_2S_2O_4$ and over prolonged periods of incubation (cf. Nicholls and Schonbaum, 1963), a result which was also confirmed experimentally in this study.

Reduction of Iron and Iron–Sulfur Proteins. The iron protein, rubredoxin, from *Peptostreptococcus elsdenii* was also reduced rapidly by trace amounts of deazaflavin in the photochemical system (this work, results not shown). Several iron–sulfur proteins have also been shown to be reduced rapidly. H. Grande, J. Fee, and R. H. Sands (personal communication) have shown rapid photoreduction of the 8 Fe iron–sulfur protein of *Micrococcus aerogenes* and the 8-iron ferredoxin from *Chromatium*. These proteins have very low redox potentials and are reduced with difficulty by dithionite, which is also required in excess for full reduction. Scherings et al. (1977) have also employed our photochemical method for the reduction of two iron–sulfur proteins isolated from *Azotobacter vinelandii*. The molybdo iron–sulfur protein, deflavoxanthine

oxidase, is also readily photoreduced by the deazaflavin–EDTA system (L. Siconolfi and V. Massey, unpublished results).

Failure of Deazaflavin to Act as a Catalyst in the Photoreduction of Deazaflavoproteins. In recent years the deazaflavins, deaza-FMN and deaza-FAD, have been bound to the apoproteins of various flavo-enzymes, and used as probes for mechanistic studies (Edmondson et al., 1972; Jorns and Hersh, 1975; Hersh and Jorns, 1975; Fisher et al., 1976; Averill et al., 1975; Cromartie and Walsh, 1976; Abramovitz and Massey, 1976). In all cases these artificial enzymes are reduced only with difficulty by substrates. In view of the facile nature of the photoreduction of the native enzymes by trace amounts of deazaflavin, it was with surprise that we found this system in general incapable of reducing deazaflavoproteins. The following were tried: deazariboflavin bound to egg white riboflavin binding protein, deaza-FMN bound to apo-lactate monooxygenase, and to apoflavodoxin. The only exception was with deaza-FAD bound to apo-D-amino acid oxidase. This artificial enzyme has been shown previously by Hersh et al. (1976) to be converted to a stable radical species by EDTA and light. Similar results were obtained in the present study; the addition of catalytic amounts of $dRFl_{ox}$ or dFl_{ox} resulted in a marked enhancement of the photoreduction rate.

General Discussion

From the results presented it is obvious that the photoreaction system described provides a general method for reduction of oxidizing enzymes, including flavoproteins, cytochromes, peroxidases, and iron–sulfur proteins. By controlling the light intensity and illumination period, flavoproteins can be converted in general either to their semiquinoid or fully reduced forms. The amounts of deazaflavin required as catalyst are sufficiently low as not to interfere with the determination of the absorption spectra of these forms, and the method is sufficiently rapid and mild that it does not result in losses of enzyme activity.

It is also evident that the principle of the photoreductive process is different from that operating with free flavins as catalysts (Massey et al., 1978). With normal flavins the photochemical process involves reduction of the flavin followed by a dark reaction with the flavoprotein. With deazaflavins the product of the photoreaction is not 1,5-dihydrodeazaflavin, but a radical dimer (see Figures 3–5). Such a dimer has been isolated and characterized by NMR spectroscopy in the case of 3,10-methyl-5-deazaflavin (see Appendix). Substantive experimental evidence for dimer formation with deazariboflavin is also given in the present paper by the finding that the major products of the EDTA–deazariboflavin light irradiation react rapidly with ferricyanide, with the consumption of 1 equiv of $Fe(CN)_6^{3-}$ per equiv of $dRFl_{ox}$ produced (Figure 4).

The formation of NAD-dimer when NAD^+ is irradiated with visible light in the presence of EDTA and trace amounts of dFl_{ox} is strong presumptive evidence for the involvement of dFlH° in the photochemical reaction. Most chemical reductions of NAD^+, such as by dithionite or borohydride, are two-electron reduction processes, and result in the formation of a mixture of dihydro-NAD species, including the enzymically active form, NADH (Yarmolinsky and Colowick, 1956; Wallenfels and Gellrich, 1959). No NAD dimer has ever been detected in such reductions. On the other hand, reductions involving one-electron reducing equivalents, such as pulse radiolysis or electrolysis, result in NAD dimers, which are enzymically inactive, and nonfluorescent (Burnett and Underwood, 1968; Land and Swallow, 1968; Schmakel et al., 1975). These dimers, which are thought to be 4,4′ dimers

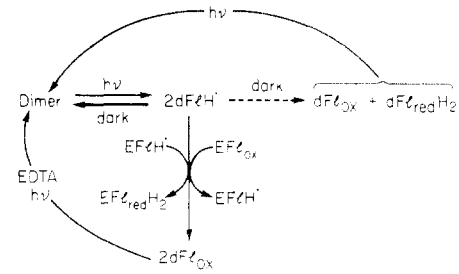

FIGURE 9: Hypothetical scheme to explain the catalytic effects of deazaflavins in photochemical reductions.

(Burnett and Underwood 1968; Schmakel et al., 1975), result from the rapid second-order self reaction of NAD°, with rate constants of the order of 10^6 to 10^7 M^{-1} s^{-1} (Land and Swallow, 1968; Schmakel et al., 1975).

$$NAD^+ \xrightarrow{e^-} NAD^\circ$$

$$2NAD^\circ \longrightarrow (NAD)_2$$

To these reactions can also be added the observed re-formation of NAD^+ from the dimer by oxidation with ferricyanide.

$$(NAD)_2 + 2Fe(CN)_6^{3-} \longrightarrow 2NAD^+ + 2Fe(CN)_6^{3-}$$

The formation of NAD dimer in the photochemical system described can therefore be explained by the intermediate production of dFlH° in the light reaction and its reaction with NAD^+.

$$dFlH^\circ + NAD^+ \longrightarrow dFl_{ox} + NAD^\circ + H$$

A rational scheme to explain the catalytic role of deazaflavins in the photoreductions is given in Figure 9. The dimeric nature of the major product of the light reaction is given by ferricyanide titration experiments such as that shown in Figure 4. That the dimer requires further light irradiation in order to produce the active reductant is demonstrated by the failure of the EDTA–light product to react with flavodoxin and other flavoproteins in the dark, and the extreme ease of reduction on further irradiation. The product of the secondary irradiation is presumed to be the deazaflavin radical, as evidenced by the production of NAD–radical dimer when NAD^+ is subjected to the photochemical system. The photochemical action spectrum (Figure 2) clearly contains contributions both from the deazaflavin dimer and dFl_{ox}. Thus the photodissociation of dimer is presumably also promoted by excited dFl_{ox} molecules.

$$(dFlH)_2 + dFl_{ox}^* \longrightarrow 2dFlH^\circ + dFl_{ox}$$

By analogy with pyridinium radicals, re-formation of dimer in the dark by self-reaction of the deazaflavin radicals is also envisaged. An alternative route for decay of the deazaflavin radicals would be the dismutation reaction shown. Any radicals escaping oxidation–reduction reactions by this route would, however, be returned to the dimer by the observed rapid photochemical generation of dimer from equimolar mixtures of dFl_{ox} and $dFl_{red}H_2$ in the absence of EDTA.

It should be noted that a long-wavelength absorbing species, presumed to be radical ($\lambda_{max} \sim 530$ nm), was observed by Edmondson et al. (1972) as a transient intermediate in flash-photolysis experiments with deazariboflavin. In our photochemical system we therefore envisage the reactive species to be the radical, dFlH°. Polarographic studies have shown that the couple $dFl_{ox}/dFlH°$ has a very low redox potential, approximately −0.65 V at pH 7.0 (Blankenhorn, 1976). Hence dFlH° is a very powerful one-electron reductant. This property explains nicely the ease of deazaflavin-photocatalyzed reduction of low potential proteins, such as the flavodoxins and iron-sulfur proteins, some of which are reduced only with high concentrations of dithionite.

The failure of the deazaflavin photochemical system to reduce the glucose oxidase and lactate monooxygenase anionic radicals could possibly be due to ionization of the deazaflavin radical in the neutral pH range ($dFlH° \rightleftharpoons dFl°^- + H^+$) and lack of reactivity of $dFl°^-$ with the negatively charged flavoprotein anionic radical. If this were the explanation, then the behavior of the red radical species of D-amino acid oxidase, L-amino acid oxidase, and Old Yellow Enzyme, which are reduced, has to be accounted for. The possibility exists that these are in fact not anion radicals but red-colored neutral radicals. It has been shown (Muller et al., 1971) that the flavin species $Fl°^-$, 1-RFl°, and 2α-RFl° (R being an alkyl residue) have similar absorption spectra, in contrast to the blue isomer 5-RFl°. Hence red-colored flavoprotein semiquinones can only safely be ascribed to the anion, $EFl°^-$, when reversible protonation to the blue EFlH° is observed on lowering the pH. In all other cases, the red species might be either $EFl°^-$ (with the p*K* shifted to acid values by the apoprotein), E·1-HFl° or E·2α-HFl°. The two latter species would be expected to be reduced by $dFl°^-$, whereas the true anion $EFl°^-$ would be expected to be reduced with difficulty, if at all. The fact that glucose oxidase is established to have a p*K* of 7.5 for the transition, $EFlH° \rightleftharpoons EFl°^- + H^+$, and is reduced readily by the deazaflavin photochemical system at low pH values but not at high pH values is suggestive that this explanation may be correct.

The inability of the deazaflavin photochemical system to reduce deazaflavoproteins may be due simply to a thermodynamic barrier. It is not unusual to find that, on binding to apoproteins, the redox potential of the normal flavin coenzyme is lowered considerably, due to weaker binding of the reduced flavin than of the oxidized form. If the deazaflavin radical were to have a weaker binding constant to the apoprotein than the oxidized form, then the redox potential of the couple $E·dFl_{ox}/E·dFlH°$ could be lower than that of the free system $dFl_{ox}/dFlH°$, and reduction could be unfavorable on thermodynamic grounds. The results with deaza-D-amino acid oxidase are also consistent with this hypothesis. This is so far the only case where radical stabilization has been observed. The redox potential of the couple $E·dFl_{ox}/EdFl_{red}H_2$ has been estimated to be −0.240 V (Jorns and Hersh, 1976). As the redox potential of the free deazaflavin couple $dFl_{ox}/dFl_{red}H_2$ has been determined to be −0.275 V (Stankovich and Massey, 1976), it can be concluded that $dFAD_{red}H_2$ is in fact bound more tightly to apo-D-amino acid oxidase than dFAD. In view of the finding with dFAD-D-amino acid oxidase that substantial amounts of the radical form accumulate on light irradiation in the presence of EDTA (Hersh et al., 1976), it can also be concluded that the redox potential of the couple $dFl_{ox}/dFlH°$ must be in the neighborhood of −0.240 V (or even more positive). Hence, in this case a thermodynamic barrier to reaction with free dFlH° would not exist, in keeping with our observation of catalysis of the photoreaction by dFl_{ox}.

In mechanistic studies on flavoproteins where deazaflavins have been employed as replacements for the natural coenzymes, it is therefore important to realize the fundamental differences in redox properties which exist between flavins and deazaflavins. By virtue of the comparatively narrow difference in potential between the couples $Fl_{ox}/FlH°$ and $FlH°/Fl_{red}H_2$ with E_0, pH 7, values of −0.24 V and −0.172 V, respectively (Draper and Ingraham, 1968), the flavin radical state is stabilized and flavins may participate in both $1e^-$ and $2e^-$ transfers. By differential binding affinity to specific apoproteins, the thermodynamic stability of the flavin radical is generally enhanced, as evidenced by the numerous examples where quantitative or near quantitative concentrations of flavoprotein radicals can be obtained (cf. Massey and Palmer, 1966). By contrast, with deazaflavins the difference in redox potentials between the couples $dFl_{ox}/dFlH°$ and $dFlH°/dFl_{red}H_2$ is enormously widened, resulting in the thermodynamic destabilization of the radical state. Thus, with pH 7.0 mid-point potentials of −0.275 V for the overall couple $dFl_{ox}/dFl_{red}H_2$ (Stankovich and Massey, 1976) and −0.650 V for $dFl_{ox}/dFlH°$ (Blankenhorn, 1976), the potential of the remaining couple $dFlH°/dFl_{red}H_2$ can be estimated as +0.100 V. Hence it is clear that a considerable thermodynamic barrier exists to $1e^-$ transfers to deazaflavin, unless, as with the one known example of D-amino acid oxidase, the potential of the couple $dFl_{ox}dFlH°$ is moved to a more favorable value by preferential affinity of the apoprotein for the radical form. The finding with other deazaflavoproteins (this paper) that reduction does not occur even when the proteins are presented with the low potential dFlH° indicates that on binding of deazaflavins to these apoproteins the potential is made even less favorable. Thus it is not surprising that of the three physiological roles of flavins, transhydrogenation, oxygen activation, and electron transfer, only transhydrogenation is exhibited with deazaflavoproteins. Even this activity is generally slowed by a factor of at least 1000 compared with that exhibited by the notive flavoprotein.

Finally some comment should be made on the finding that protein-bound flavin and a wide variety of iron-sulfur and heme centers are so easily accessible to reaction with free flavins and deazaflavins. It will be the aim of further studies to elucidate the scope and limitations of the deazaflavin photochemical system. There appears to be at least one iron-sulfur protein in the nitrogenase complex of *Azotobacter vinelandii* which is not accessible to reduction by dFlH°, but which requires the intermediary catalyst flavodoxin, which is reduced by dFlH° (Scherings et al., 1977). The possibility therefore exists that deazaflavins may serve as suitable probes for the structural accessibility of iron-sulfur centers and perm[illegible] a further categorization within this group of redox protein[illegible]

Acknowledgments

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References

Abramovitz, A., and Massey, V. (1976), *J. Biol. Chem. 251,* 5327-5336.

Averill, B. A., Schonbrunn, A., Abeles, R. H., Weinstock, L. T., Cheng, C. C., Fisher, J., Spencer, R., and Walsh, C. (1975), *J. Biol. Chem. 250,* 1603-1605.

Blankenhorn, G. (1976), *Eur. J. Biochem. 67,* 67-80.

Burnett, R. W., and Underwood, A. L. (1968), *Biochemistry*

7, 3328-3333.
Cromartie, T. H., and Walsh, C. (1976), *J. Biol. Chem. 251,* 329-333.
Draper, R. D., and Ingraham, L. L. (1968), *Arch. Biochem. Biophys. 125,* 802-808.
Edmondson, D. E., Barman, B., and Tollin, G. (1972), *Biochemistry 11,* 1133-1138.
Enns, K., and Burgess, W. H. (1966), *J. Am. Chem. Soc. 87,* 5766-5770.
Fisher, J., Spencer, R., and Walsh, C. (1976), *Biochemistry 15,* 1054-1064.
Frisell, W. R., Chung, C. W., and Mackenzie, C. G. (1959), *J. Biol. Chem. 234,* 1297-1302.
Frost, A. A., and Pearson, R. G. (1961), Kinetics and Mechanism, New York, N.Y., Wiley, pp 162-164.
Hersh, L. B., and Jorns, M. S. (1975), *J. Biol. Chem. 250,* 8728-8734.
Hersh, L. B., Jorns, M. S., Peterson, J., and Currie, M. (1976), *J. Am. Chem. Soc. 98,* 865-867.
Iyanagi, T., Makino, N., and Mason, H. W. (1974), *Biochemistry 13,* 1701-1710.
Janda, M., and Hemmerich, P. (1976), *Angew. Chem. 15,* 443-444.
Jorns, M. S., and Hersh, L. B. (1975), *J. Biol. Chem. 250,* 3620-3628.
Jorns, M. S., and Hersh, L. B. (1976), *J. Biol. Chem. 251,* 4872-4881.
Komai, H., Massey, V., and Palmer, G. (1969), *J. Biol. Chem. 244,* 1692-1700.
Land, E. J., and Swallow, A. J. (1968), *Biochim. Biophys. Acta 162,* 327-337.
Massey, V., and Ghisla, S. (1975), *Fed. Eur. Biochem. Soc., 10th Meet.,* 145-158.
Massey, V., and Hemmerich, P. (1977), *J. Biol. Chem. 252,*5612-5614.
Massey, V., and Palmer, G. (1966), *Biochemistry 5,* 3181-3189.
Massey, V., and Veeger, C. (1961), *Biochim. Biophys. Acta 48,* 33-47.
Massey, V., Gibson, Q. H., and Veeger, C. (1960), *Biochem. J. 77,* 341-351.
Massey, V., Stankovich, M., and Hemmerich, P. (1978), *Biochemistry 17* (preceding paper in this issue).
Müller, F., Hemmerich, P., and Ehrenberg, A. (1971), in H. Kamin, Ed., Flavins and Flavoproteins, Baltimore, Md., University Park Press, pp 107-122.
Nicholls, P., and Schonbaum, G. R. (1963), *Enzymes, 2nd Ed., 8,* 147-225.
Peterson, J. A., White, R. E., Yasukochi, Y., Coombs, M. L., O'Keefe, D. H., Ebel, R. E., Masters, B. S. S., Ballou, D. P., and Coon, M. J. (1977), *J. Biol. Chem. 252,* 4431-4434.
Scherings, G., Haaker, H., and Veeger, C. (1977), *Eur. J. Biochem.* (in press).
Schmakel, C. O., Santhanam, K. S. V., and Elving, P. J. (1975), *J. Am. Chem. Soc. 97,* 5083-5092.
Spencer, R., Fisher, J., and Walsh, C. (1976), *Biochemistry 15,* 1043-1053.
Spector, T., and Massey, V. (1972), *J. Biol. Chem. 247,* 5632-5636.
Stankovich, M. T., and Massey, V. (1976), *Biochim. Biophys. Acta 452,* 335-344.
Stein, G., and Swallow, A. J. (1958), *J. Chem. Soc.,* 306-312.
Strickland, S., and Massey, V. (1973), *J. Biol. Chem. 248,* 2944-2952.
Vermilion, J. L., and Coon, M. J. (1977), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 959.
Wallenfels, K., and Gellrich, M. (1959), *Ber. 92,* 1406-1415.
Yarmolinsky, M. B., and Colowick, M. B. (1956), *Biochim. Biophys. Acta 20,* 177-189.

Appendix: Photochemical Formation of Deazaflavin Dimers

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The structure of the photochemical product of deazaflavin as a 5,5′ dimer (cf. Figure 5) is shown by the following NMR analyses. 5,5′-Bis(3,10-dimethyl-1,5-dihydro)-5-deazaisoalloxazine was prepared by photolysis under anaerobic conditions of 500 mg (0.0021 mol) of 3,10-dimethyl-5-deazaisoalloxazine with 4.5 g (0.05 mol) of sodium oxalate in a volume of 300 mL, with pH adjusted to 8. Illumination was carried out with a water-cooled high pressure mercury immersion lamp (Philips HPK 125W). The progress of the reaction was monitored by withdrawing aliquots and measuring the remaining oxidized deazaflavin by its absorbance at 393 nm. After 2 h there was no remaining oxidized deazaflavin. The solution was then adjusted to pH 4.0 with acetic acid. The precipitated product was filtered, washed with methanol and ether, and then dried in vacuum at 50 °C over P_2O_5. The dried product was dissolved in carbonate buffer (pH ~10) prepared in ^2H_2O. ^1H NMR spectra were determined with a Varian A60A analytical spectrometer at ambient temperature, using an internal standard of sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)-propanesulfonate. Figure 10 shows the results for the product

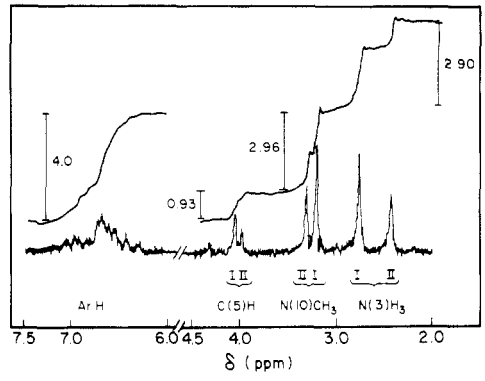

FIGURE 10.

isolated as described. The aromatic protons absorb in the range 6-7.2 ppm. Setting the integration in this region equal to 4 protons, the remaining signals may be assigned to the C(5), $N(10)CH_3$, and $N(3)CH_3$ protons as shown. This assignment is in agreement with that of Brustlein and Bruice (1972) for 3,10-dimethyl-1,5-dihydro-5-deazaisoalloxazine. It is clear from Figure 10 that there are at least two products, labeled I and II, in the proportions of 6:4. The two signals at 4.03 ppm and 4.10 ppm, associated with C(5)H, correspond to only a single proton, proving the dimeric structure of the product.

The formation of a mixture of products is not surprising,

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because a 5,5′ dimer would have two chiral centers. Hence the analogous stereochemistry would be expected to be that found for tartaric acid, i.e., two enantiomers and a meso form. These three isomers presumably show two different ^{1}H NMR spectra because the enantiomers have the same chemical shifts and different from those of the meso form. The chemical shifts are as follows (dimer I): N(3)CH_3, 2.80, N(10)CH_3, 3.25, C(5)H, 4.10, ArH, 6.28–7.28 ppm; (dimer II) N(3)CH_3, 2.47, N(10)CH_3, 3.35, C(5), 4.03, ArH, 6.28–7.28 ppm.

Interconversion of the dimers is possible. If the irradiation is continued for 8 h before isolation, the ^{1}H NMR spectrum is almost pure dimer II. If, on the other hand, the mixture is incubated anaerobically in 6 N HCl, the ^{1}H NMR spectrum is almost pure dimer I. The two dimers have nearly identical absorption spectra, but they can be distinguished also on the basis of their rates of reoxidation with ferricyanide. At pH 8.6, 25 °C, dimer I is converted to the oxidized deazaflavin at a rate of 2.5×10^4 M^{-1} s^{-1}; the corresponding rate for dimer II is 7.2×10^3 M^{-1} s^{-1}. Because of the rapidity of this reoxidation, titration with $K_3Fe(CN)_6$ yields accurate information on the oxidation–reduction state of the isolated compounds. With both I and II exactly 1 equiv of $K_3Fe(CN)_6$ was consumed per equiv of oxidized deazaflavin formed, providing definitive evidence for their dimeric structure. This work will be reported in full elsewhere.

Reference

Brustlein, M., and Bruice, T. C. (1972), *J. Am. Chem. Soc. 94,* 6548.

Sedimentation Behavior of Native and Reduced Apolipoprotein A-II from Human High Density Lipoproteins†

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ABSTRACT: The solution properties of human serum apolipoprotein A-II, both in the native and in the reduced forms, were investigated by the technique of sedimentation equilibrium in the analytical ultracentrifuge. For both proteins, the apparent weight average molecular weights determined in neutral buffer systems were found to be dependent on protein concentration and invariant with the rotor speeds used (16 000 to 44 000 rpm) indicating a reversible self-association. These results were also found to be independent of temperature between 5 and 30 °C. The pattern of self-association of native apolipoprotein A-II could best be described by a monomer–dimer–trimer equilibrium, in agreement with previously reported data (Vitello, L. B., and Scanu, A. M. (1976), *Biochemistry 15,* 1161). The self-association pattern of apolipoprotein A-II reduced in the presence of 50 mM dithiothreitol conformed with a monomer–dimer–tetramer equilibrium similar to that reported for the native single chain apolipoprotein A-II of the rhesus monkey (Barbeau, D. L., et al. (1977), *J. Biol. Chem. 252,* 6745), but differing significantly from that reported for the reduced and carboxymethylated human product (Osborne, J. C., et al. (1975), *Biochemistry 14,* 3741).

Apolipoprotein A-II (apo-A-II),[1] one of the major protein components of human high density lipoprotein (HDL) (Morrisett et al., 1975; Scanu et al., 1975), contains two identical polypeptide chains linked together by a disulfide bond located in position 6 from the amino-terminal residue (Brewer et al., 1972). Recent reports from this and other laboratories have shown that human apo-A-II in its native unreduced form undergoes self-association in aqueous solutions. However, the reported modes of self-association show no agreement (Vitello and Scanu, 1976; Stone and Reynolds, 1975; Gwynne et al., 1975).

Recent studies from this laboratory (Barbeau et al., 1977) have indicated that the native single chain apo-A-II from the rhesus monkey undergoes self-association in solution, and that the mode of association differs from that reported for the reduced and carboxymethylated human apo-A-II (Cm-apo-A-II) (Osborne et al., 1975). These observations led us to suspect that the carboxymethyl group might influence the self-association of the single-chain apo-A-II. Based on the above information, we felt it appropriate to reinvestigate the solution properties of native apo-A-II in the ultracentrifuge and to compare the results with those of the corresponding single-chain protein in the presence of the reducing agent, dithiothreitol. In this report, we will present the results of sedimentation equilibrium studies which were carried out over a wide range of initial protein concentrations and temperatures.

Materials and Methods

Preparation and Purification of Apo-A-II. The HDL of density 1.063–1.21 g/mL were separated from fresh human sera by cumulative ultracentrifugal flotation as previously

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[1] Abbreviations used: HDL, high density lipoproteins of density 1.063 to 1.210 g/mL; apo-HDL, delipidated HDL; apo-A-I, apolipoprotein A-I; apo-A-II, apolipoprotein A-II; Cm-apo-A-II, reduced and carboxymethylated apo-A-II; EDTA, ethylenediaminetetraacetic acid; DEAE diethylaminoethyl; RMS, root mean square.

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