On the Importance of the N-5 Position in Flavin Coenzymes. Properties of Free and Protein-Bound 5-Deaza Analogs†

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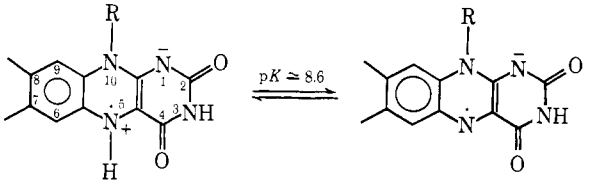
ABSTRACT: As a means of providing information on the role of the 5-position nitrogen atom in determining the properties of free and protein-bound flavin coenzymes, we have examined the spectral and redox behavior of 5-deaza analogs both in solution and bound to the apoprotein of the Shethna flavoprotein from *Azotobacter*. The absorption, fluorescence, and circular dichroism (CD) spectra of these analogs are quite similar to those of ordinary flavins. Dithionite reduces the deazaflavins very slowly in a two-electron process which generates a fully reduced analog. This derivative is considerably more resistant to oxidation than is the corresponding reduced flavin. The deaza analogs can be photoreduced with EDTA and flash photolysis demonstrates the formation of triplet states and semiquinone radicals. Unlike ordinary flavins, mixtures of oxidized and fully reduced deazaflavins do not contain measurable amounts of either semiquinone or $F \cdot FH_2$ complex. Both 5-deazariboflavin and 5-deazaFMN bind readily to the apoprotein of the Shethna flavoprotein. Measurements of binding rates, association constants, and CD spectra of the analog flavoproteins indicate that the 5 position is important in flavin binding. Unlike the native flavoprotein, the deazaFMN protein is *fully* reduced either by dithionite or light plus EDTA, indicating that the 5-position nitrogen is crucial in allowing the protein to stabilize the half-reduced form of the flavin coenzyme.

One of the more interesting questions in enzymology which remains largely unanswered is the following. By what means do the interactions between an apoprotein and its coenzyme modify the properties of the coenzyme so as to adapt it to its specific biochemical function? Flavoproteins provide a convenient system to use for investigating this problem because of the broad range of metabolic processes in which they participate, involving a wide variety of substrates and oxidants. Furthermore, in many cases the coenzyme can be reversibly removed from the flavoprotein, allowing kinetic and thermodynamic studies of the binding process. Finally, the chemical and spectroscopic properties of the flavin isoalloxazine ring system have been thoroughly studied, and thus comparisons between free and bound coenzyme can be conveniently made.

Previous work (Holmström, 1964; Vaish and Tollin, 1971) has shown that the nitrogen atom in the 5 position of the isoalloxazine ring is involved in determining some of the properties of the half-reduced (semiquinone radical) form of the coenzyme. For example, the state of protonation at this position controls the reactivity of the radical toward oxygen



neutral radical; unreactive toward O_2

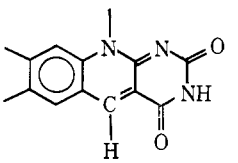
anion radical; highly reactive toward O_2

In addition, metal-free flavoproteins show a high degree of specificity with respect to the type of radical which they generate upon partial reduction, the dehydrogenases generally forming the neutral (blue) semiquinone and the oxidases the anionic (red) semiquinone (Massey *et al.*, 1969). It would appear, then, that one of the ways in which the reactivity of the flavin coenzyme is controlled involves the type of protein interaction at the N-5 position, which either stabilizes or destabilizes the protonation of the radical form (Müller *et al.*, 1970) and thus influences its ability to be oxidized by O_2.

Another indication that the 5 position of the isoalloxazine structure is significant in determining the properties of flavoenzymes is to be found in the fact (Massey *et al.*, 1969; Müller and Massey, 1969) that flavoprotein oxidases readily form adducts with sulfite ion at the N-5 position whereas dehydrogenases do not. Thus, either the accessibility and/or the reactivity of this position is different in these two types of flavoenzymes.

The present work constitutes a further exploration of the importance of the N-5 position in the flavin system. We have utilized the 5-deaza analogs[1] (O'Brien *et al.*, 1970) of FMN and riboflavin and have compared the properties of the free compounds to those of a flavoprotein derivative produced by binding 5-deazaFMN to the apoprotein of the Shethna flavoprotein from *Azotobacter*. The latter was chosen because it forms the most stable half-reduced species of all known flavoproteins (Hinkson and Bulen, 1967; Edmondson and Tollin, 1971a–c).

[1] In these compounds the nitrogen at position 5 is replaced by CH



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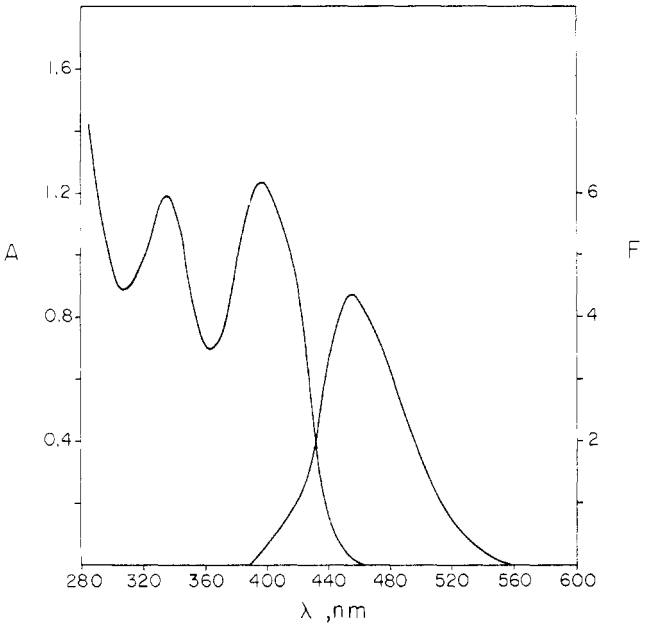


FIGURE 1: Absorption and fluorescence emission spectra of 5-deazariboflavin in 0.025 M phosphate buffer (pH 7.0). The fluorescence spectrum is uncorrected for monochromator efficiency and detector response.

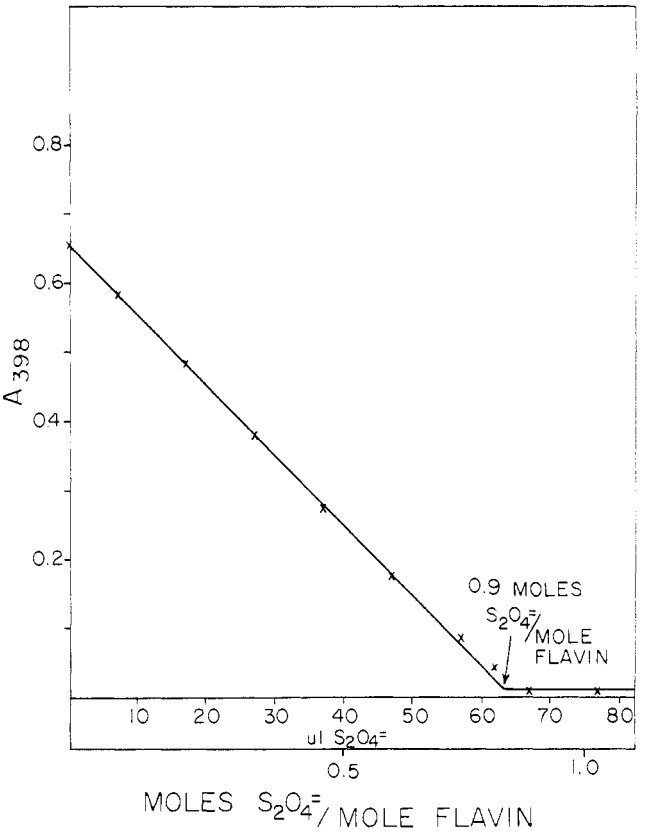


FIGURE 2: Anaerobic dithionite titration of 5-deazaFMN in 0.1 M phosphate buffer (pH 7.2).

Experimental Section

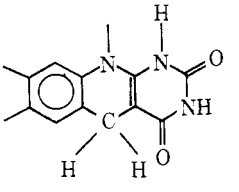
5-Deazariboflavin was a gift from Dr. C. C. Cheng, Midwest Research Institute, Kansas City, Mo., with the authorization of Drug Research and Development, Chemotherapy, of the National Cancer Institute. This compound was phosphorylated according to the method of Flexser and Farkas (1952) to give 5-deazaFMN, which was purified by DEAE-cellulose column chromatography. The Shethna flavoprotein from *Azotobacter vinelandii* was isolated by the procedure of Hinkson and Bulen (1967) and the apoprotein was prepared as described by Edmondson and Tollin (1971b). Cytochrome *c* (horse heart) was obtained from Sigma (A grade). All other materials were reagent grade from standard suppliers.

Absorption spectra were determined using a Cary Model 14R or a Coleman Model 124 spectrophotometer. Circular dichroism (CD) spectra were obtained with a Cary Model 60 spectropolarimeter. Fluorescence measurements were made with a previously described instrument (D'Anna and Tollin, 1971). Flash photolysis experiments were carried out as outlined in an earlier paper (Vaish and Tollin, 1970). A dithionite titration of deazaFMN was performed according to the method of Foust *et al.* (1969). All other techniques were as described earlier (Edmondson and Tollin, 1971b,c; D'Anna and Tollin, 1971; Vaish and Tollin, 1970).

Results and Discussion

The visible absorption (O'Brien *et al.*, 1970) and fluorescence emission spectra of free 5-deazariboflavin in aqueous solution are shown in Figure 1. The wavelength maxima for both types of spectra are shifted 50–60 nm to the blue as compared with ordinary riboflavin. Although no quantitative determinations of fluorescence quantum yield were made, visual comparison indicated that this quantity is similar in magnitude to that for riboflavin.

The 5-deaza analogs were found to have redox properties qualitatively similar to those of ordinary flavins. However, sodium dithionite reduction proceeds considerably more slowly than with flavins. A plot of the pseudo-first-order rate constant in the presence of 0.8×10^{-5} M dithionite as a function of deazaFMN concentration was linear, and gave a calculated second-order rate constant of 3.8×10^{3} M^{-1} min^{-1} (the half-time for the reduction at a deazaflavin concentration of 2.2×10^{-5} M was 9 min, as compared to less than 1 sec for FMN). Titration experiments (Figure 2) showed that two electrons were added per molecule of deazaflavin, presumably to form a fully reduced derivative



The fully reduced deaza compound is much more resistant to oxidation than is the corresponding reduced flavin. Thus, O_2 oxidation of dithionite-reduced material,[2] measured at room temperature in air-saturated 0.1 M Tris buffer (pH 8.4) proceeded with a pseudo-first-order rate constant of 0.0038 min^{-1} ($\tau_{1/2}$ = 180 min). This can be compared to a half-time of less than 1 sec (Gibson and Hastings, 1962) for $FMNH_2$. Similarly, oxidation of reduced deazariboflavin by horse heart cytochrome *c* (1.7×10^{-5} M–0.1 M Tris buffer (pH 8.4)) occurred with a pseudo-first-order rate constant of 0.02 min^{-1}

[2] Illumination with visible light was found to markedly accelerate the rate of air oxidation.

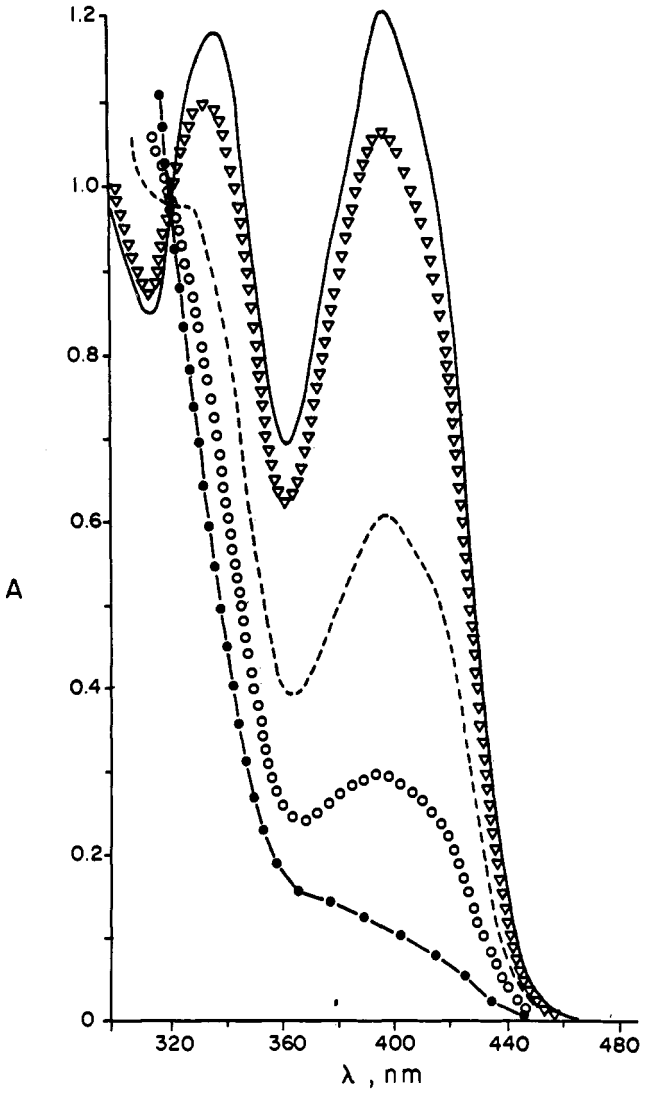


FIGURE 3: Photoreduction of 5-deazariboflavin in 0.1 M Tris buffer (pH 8.4), containing 0.1 M EDTA. (—) Fully oxidized; (●) fully reduced. Other curves represent intermediate stages of reduction.

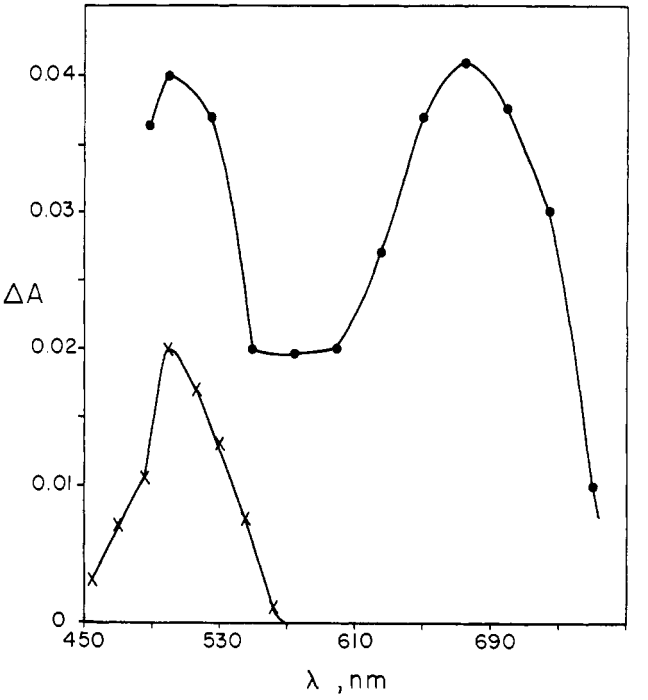


FIGURE 4: Flash-induced difference spectra for 5-deazariboflavin in 0.025 M acetate buffer (pH 4.8). (×) [flavin] = 8×10^{-6} M; [3,4-dimethylphenol] = 1×10^{-3} M; (●) [flavin] = 1.5×10^{-5} M.

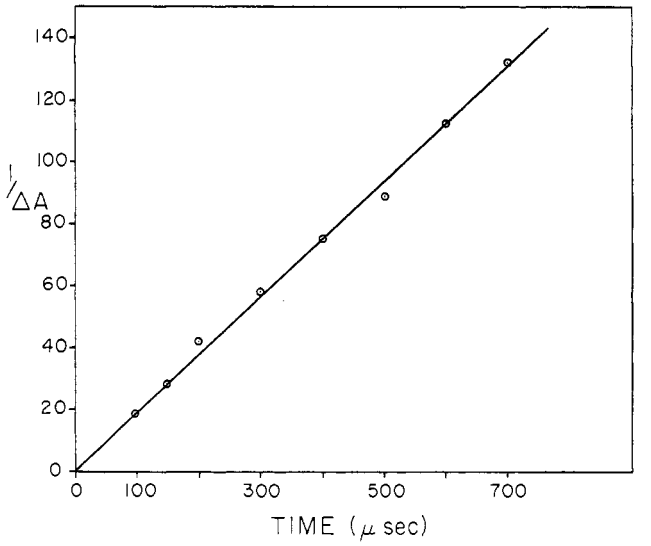


FIGURE 5: Second-order plot of decay of flash-induced transient at 500 nm for 5-deazariboflavin in 0.025 M acetate buffer (pH 4.8), containing 0.05 M EDTA.

($\tau_{1/2}$ = 35 min), compared to a half-time of about 15 sec for $FADH_2$ (Dixon, 1971). These slower oxidation rates are probably a consequence of the loss of the nonbonded electron pair in going from nitrogen to CH at position 5 and thus a lower availability of electrons.

The deazaflavin analogs undergo photoreduction in the presence of EDTA at a rate which is at least as fast as that of ordinary flavins. Figure 3 shows a series of absorption spectra taken at various stages of photoreduction. An isosbestic point occurs at 333 nm (compared to 330 nm for FMN (Beinert, 1960)). A similar isosbestic point was observed in the dithionite reduction and subsequent air oxidation (another isosbestic point at 290 nm was also obtained).

The ability of the deaza analogs to be photoreduced implies the intermediacy of triplet states and semiquinone radicals (Vaish and Tollin, 1970). In order to confirm this, a flash photolysis study was performed. In Figure 4, flash-induced difference spectra are shown for 5-deazariboflavin in the presence and absence of 3,4-dimethylphenol (Vaish and Tollin, 1970). The transient which has an absorption maximum at approximately 500 nm is probably the neutral semiquinone radical (the maximum wavelength for the corresponding FMN semiquinone is about 560 nm; thus, the spectral shift is similar in magnitude to what was observed for the oxidized form). This identification was confirmed by a flash experiment using EDTA as the reducing agent (Vaish and Tollin, 1971). Second-order decay kinetics were observed (Figure 5) and the sample was bleached after about ten flashes. A second-order rate constant of 9.1×10^9 M^{-1} sec^{-1} was calculated,[3] which is about eight times that observed with ribo-

[3] This assumes that the absorptivity of the deaza radical is the same as that for the flavin radical (Land and Swallow, 1969).

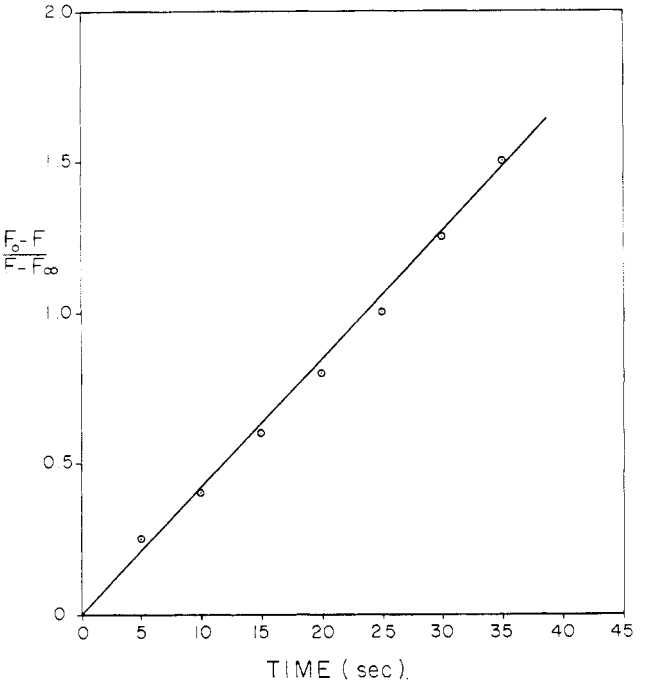


FIGURE 6: Second-order plot of fluorescence quenching accompanying binding of 5-deazaFMN to the Shethna apoprotein in 0.025 M phosphate buffer (pH 7.0). [5-deazaFMN] = 1×10^{-6} M; [apoprotein] = 1×10^{-6} M.

flavin (Land and Swallow, 1969), about three times that for FMN and slightly larger than for lumiflavin (Vaish and Tollin, 1971). Qualitatively, the radical yield was somewhat smaller for the deaza analog than for lumiflavin. As with ordinary flavins, sample bleaching by the flash was considerably retarded when phenol was used as the reductant (Vaish and Tollin, 1970). These experiments confirm that a neutral radical form can be generated with the deazaflavins and that disproportionation of the radical can occur to form a fully reduced compound.

The transient which has an absorption maximum at approximately 675 nm is most probably the triplet state (Vaish and Tollin, 1970; Knowles and Roe, 1968). This is confirmed by the fact that the signal was not observed in the presence of reducing agents (Figure 4), presumably because of photochemical quenching. The lifetime and yield of the deazaflavin triplet are comparable to those for ordinary flavins. This is consistent with the observed rapid rate of photoreduction.

Mixtures of approximately equal amounts of oxidized and fully reduced 5-deazaFMN (about 10^{-3} M in each component) do not contain measurable amounts of free radical or $F \cdot FH_2$ complex (as determined by absorption and electron spin resonance (esr) spectrometry). These species are easily detectable under corresponding conditions with FMN (Gibson *et al.*, 1962). Thus, radical disproportionation is essentially irreversible with the deaza analogs. It is somewhat surprising that no complex is formed; this points to an important role for the 5-position nitrogen in the stabilization of this species, perhaps *via* a key hydrogen bond.

Ordinary flavins form high yields of cation radical when dissolved in 47% HI (Fleischman and Tollin, 1965). On the basis of both esr and optical spectroscopic measurements, deazaflavins are not reduced at all in this solvent.

With the above background of information on the properties of the free deazaflavin analogs, it seemed appropriate to investigate the binding of these compounds to a flavoenzyme apoprotein and the redox behavior of the protein-bound species. The Shethna flavoprotein from *Azotobacter* is known to form an exceptionally stable flavin free radical (Hinkson and Bulen, 1967; Edmondson and Tollin, 1971c) and the general properties of this protein have been extensively studied (Hinkson and Bulen, 1967; Edmondson and Tollin, 1971b,c; D'Anna and Tollin, 1971). We therefore felt that this would provide a good model for elucidating the role of the flavin N-5 position in dehydrogenase-type flavoenzymes. Using flavin fluorescence quenching, it is possible to demonstrate that both 5-deazaFMN and 5-deazariboflavin are bound to the apoprotein of the Shethna flavoprotein. As with other flavin analogs (Edmondson and Tollin, 1971b), the binding kinetics are second order (Figure 6). The rate constant for 5-deazaFMN binding was determined to be $4.1 \pm 0.1 \times 10^4$ M^{-1} sec^{-1}. This is approximately three times slower than observed with FMN (Edmondson and Tollin, 1971; D'Anna and Tollin, 1971b). Similarly, the binding rate for 5-deazariboflavin was 1.5–2 times slower than for riboflavin. These results provide evidence that the 5 position is involved, along with the ribityl phosphate side chain (Edmondson and Tollin, 1971b; D'Anna and Tollin, 1971), in the rate-determining interaction with the protein, and suggest that the most favorable conformation of the coenzyme for binding is a folded one in which the N-5 position and the phosphate group present themselves simultaneously to the protein binding site. This in turn would indicate that the binding site in the apoprotein has a complementary shape, *i.e.*, it is a narrow cleft.[4]

It is likely that the slower binding rates observed with the deaza analogs are due either to a lower equilibrium concentration of correctly folded conformer in these species, or to a facilitation of the binding process by an N-5–protein interaction which can occur only with the normal flavin derivatives. CD spectroscopy provides a measure of ring–side-chain interaction in the free coenzyme, and this interaction plus ring–protein interactions in the protein-bound form (Tollin, 1968; Edmondson and Tollin, 1971a). It is thus of interest to obtain these spectra for the 5-deaza species. In Figure 7 is shown the CD spectra of free 5-deazaFMN and of the 5-deazaFMN·Shethna apoprotein complex. The ellipticities of the free analog are approximately the same as those found for free FMN (Tollin, 1968; Edmondson and Tollin, 1971a). A major difference occurs in the sign of the long-wavelength CD band, which is positive for the 5-deaza derivative and negative for FMN (Tollin, 1968). This indicates that, on the average, the relative positions of the ring and side chain are different in the two species.[5] It is noteworthy that the major CD band in both flavins appears at approximately the same wavelength (*ca.* 340 nm). Thus, this band, which is not observable in the ordinary absorption spectrum (Tollin, 1968), must not have an appreciable component of the transition moment at the 5 position.

The CD spectrum of the 5-deazaFMN protein is quite similar in general shape and band signs to that of the native Shethna flavoprotein. This demonstrates that the 5-deaza FMN is bound in a specific manner to the apoprotein and that the overall conformational characteristics of the flavin

[4] An alternative possibility is that the initial flavin–protein interaction triggers a conformation change in the protein which results in the generation of a cleft-like binding site (Edmondson and Tollin, 1971b,c).

[5] This assumes that the transition moment directions are similar, which is not entirely certain, although likely because of the isoelectronic relationship which exists between the analogs.

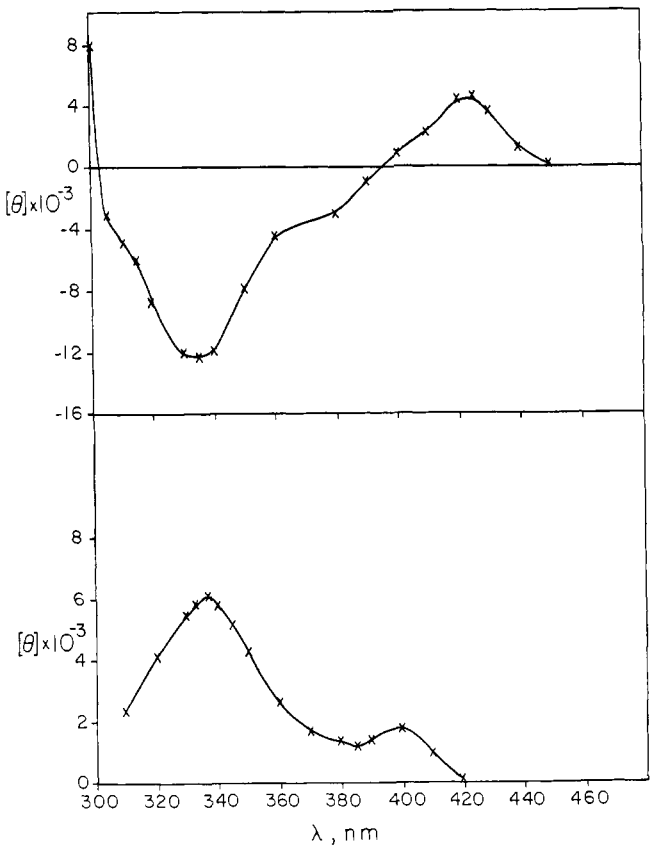


FIGURE 7: CD spectra of free 5-deazaFMN (bottom curve) and of the 5-deazaFMN·Shethna apoprotein complex (top curve) in 0.025 M phosphate buffer (pH 7.0).

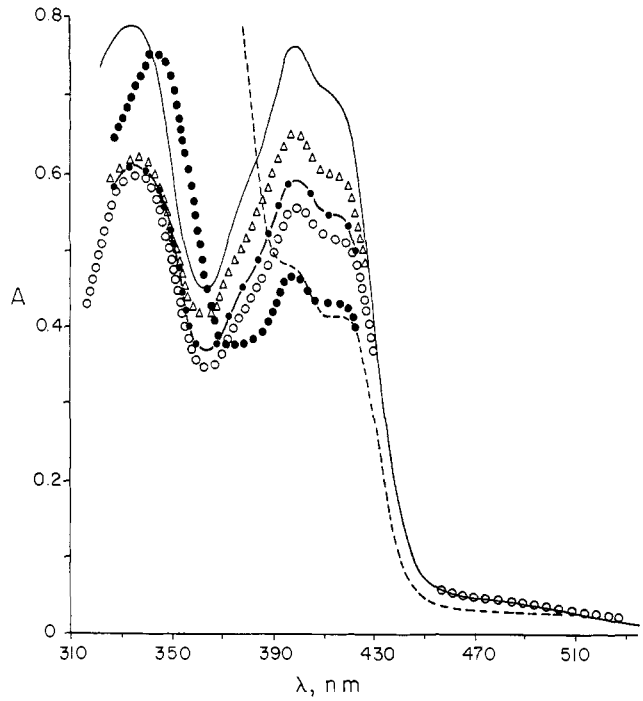


FIGURE 8: Dithionite reduction and air oxidation of 5-deazaFMN·Shethna apoprotein complex in 0.025 M phosphate buffer (pH 7.0). (—) Oxidized form; (----) dithionite reduced; (●) 25 min after O_2 added; (○) 50 min after O_2 added; (-●-) 80 min after O_2 added; (△) 140 min after O_2 added.

analog in its binding site are not too different from those of the normal coenzyme. However, the magnitudes of the ellipticities are considerably lower for the deaza analog (*ca.* 40% for the longest wavelength band and *ca.* 33% for the most intense shorter wavelength band). Inasmuch as the long-wavelength band monitors mostly the ring–side-chain interaction (Tollin, 1968; Edmondson and Tollin, 1971a) and the shorter bands the ring–protein interaction (Edmondson and Tollin, 1971a; Tollin and Edmondson, 1971), both of these characteristics can be said to be affected by the replacement of nitrogen by carbon.

The equilibrium constant for the binding of 5-deazaFMN to the Shethna apoprotein was measured to be 4×10^7 M^{-1} at room temperature (25 ± 2°). This corresponds to approximately 1 kcal/mole less binding energy than for FMN, which is not inconsistent with an N-5–protein hydrogen-bonding interaction. The fact that the binding of the deaza analog is strong suggests that the low ellipticities observed in the CD spectrum may be a consequence of a greater conformational mobility of the deaza flavin within the protein binding site caused by a steric interference of the hydrogen atom at C-5 with a protein functional group.

Sodium dithionite is capable of reducing the deazaFMN protein. However, unlike the native Shethna protein (Edmondson and Tollin, 1971c), the reaction proceeds directly to the fully reduced state at pH 7.0 with no measurable formation of the half-reduced form (Figure 8).[6] Furthermore, not all of the protein-bound flavin is reduced under these conditions. Air oxidation of the dithionite-reduced protein occurred with a half-time of about 70 min. Surprisingly, this is faster than was the case with the unbound analog. Again, no measurable radical formation occurred. With the native protein (Edmondson and Tollin, 1971c), air oxidation of the fully reduced form, which is generated by dithionite reduction above pH 8.0, produces *only* the radical species.

The deazaFMN protein is also capable of being photoreduced in the presence of EDTA. The reduction occurs slowly, as with the native protein, but *unlike* the normal flavoprotein, produces *only* the fully reduced form. Inasmuch as the photochemical reduction by EDTA is most probably a one-electron process, this suggests that the deazaflavoprotein semiquinone readily disproportionates. Upon air oxidation, the oxidized form is generated with no measurable radical production. Only about 50% of the protein can be reduced.

The fact that only partial reduction of the deazaFMN protein can be achieved, even by as strong a reducing agent as dithionite, seems to imply a thermodynamic barrier to full reduction. One possible reason for this is that there probably is a smaller dihedral angle about C-5 and N-10 for the reduced form of deazaFMN than the 150° angle for normal dihydroflavins (Kierkegaard *et al.*, 1971). Thus, the dithionite reducing potential might not be sufficient to overcome the energy barrier to stretching of the normal sp^3 carbon-bond angle from 109 to 150°. This, of course, implies that the protein geometry about the flavin binding site could not accommodate a bent flavin with a dihedral angle very much less than 150°. Previous work (Edmondson and Tollin, 1971c) indicates that even with FMN, only the anionic hydroquinone form can be accommodated in the binding site. The more rapid rate of air oxidation of the protein-bound deazaFMN hydroquinone to its planar form as compared to free deazaFMN would be consistent with this hypothesis.

[6] Note that the absorption spectrum of the oxidized form has the typical flavoprotein vibronic structuring of the long-wavelength band.

The above results demonstrate quite clearly that the N-5 position is crucial in order for the protein to be able to stabilize the half-reduced form of the flavin coenzyme. This is particularly emphasized by the fact that whereas 3-methyl-FMN and isoFMN bind to the Shethna apoprotein with approximately the same strength as does 5-deazaFMN (Edmondson and Tollin, 1971b), both of these analog proteins generate semiquinones upon either dithionite reduction or photoreduction (Edmondson and Tollin, 1971c). Although the deazaflavin radical is somewhat less stable than the normal flavin species (as judged by the faster disproportionation rate), this does not seem to be sufficient to account for the complete lack of semiquinone formation in the analog protein. The slower binding rates for the deaza analogs suggest that an important interaction occurs with the protein at the 5 position of the flavin ring. It is possible that this same interaction also participates in radical stabilization.

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A Comparison of the Nucleotide Specificity and Atractyloside Sensitivity of Digitonin and Sonic Particles†

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ABSTRACT: The specificity for phosphate acceptor of submitochondrial particles prepared by sonication and digitonin treatment was compared by use of a series of natural and synthetic analogs of ADP. The analogs examined contained changes in the purine ring, ribose, and the glycosidic linkage. The apparent K_m and V_{max} was determined for all analogs with both preparations. Contrary to previous reports, both preparations show broad specificity. The reason that digitonin particles appeared to be specific in earlier studies is that tests were conducted at a single high concentration of nucleotides at which GDP and IDP show marked substrate inhibition. The phosphate uptake with IDP as acceptor using digitonin particles was found to be inhibited by atractyloside. Two of the analogs were found to be substrates for nucleoside triphosphate synthesis with digitonin but not with sonic particles. These findings are at variance with current models of mitochondrial organization particularly with regard to the role of nucleotide translocase and the relative orientation of the vesicular membrane in digitonin and sonic particles.

Current theories on mitochondrial organization hold that ADP and ATP enter and leave the matrix compartment *via* an atractyloside-sensitive, adenine nucleotide specific translocase that is located in the inner membrane and catalyzes an exchange diffusion (Klingenberg and Pfaff, 1966; Heldt, 1967).

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‡ A portion of the data is from the thesis presented by D. C. H. in partial fulfillment of the requirements for the Ph.D. degree from Case Western Reserve University. Present address: Bristol Hospital, Bristol, Conn.