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8-Azidoflavins as Photoaffinity Labels for Flavoproteins*

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8-Azidoflavins have been synthesized and their potential as photoaffinity labels for flavoproteins has been explored. They are very photolabile, and in aqueous media they react with solvent to yield 8-aminoflavins and 8-hydroxlaminoflavins as the main products. They fulfill the criteria expected of a good photoaffinity label, since they bind stoichiometrically at the flavin-binding site of flavoproteins, thus minimizing problems of nonspecific labeling. Second, they absorb strongly in the visible, so that the reactive nitrene can be generated without short wavelength light, minimizing the possibility of light-induced damge of the protein. Third, in the absence of light, 8-N_3-flavins are stable, permitting a study of their binding to apoproteins. 8-Azidoflavins have been bound to hen egg white riboflavin-binding protein, *Megasphera elsdenii* flavodoxin, yeast Old Yellow Enzyme, *Aspergillus niger*, glucose oxidase, and pig kidney D-amino acid oxidase, and the effect of exposure to visible light has been determined. Only small extents of covalent attachment of the flavin to the protein were found with flavodoxin, D-amino acid oxidase, and Old Yellow Enzyme; much more extensive labeling was obtained with glucose oxidase and riboflavin-binding protein. In addition to their photoreactivity, 8-azidoflavins have been found to be converted to 8-aminoflavins by reaction with sulfite or upon reduction. Similar reactions occur with 8-hydroxylamino-, 8-(*O*-methyl)hydroxylamino-, and 8-hydrazinoflavins, which serve as models for possible flavin-protein covalent linkages which could be formed in the photolabeling procedure. Some of the properties of these flavins, which were obtained by reaction of 8-F-flavin with the corresponding nucleophiles, are also described.

That flavin is a highly photoactive chromophore has been recognized since Kuhn's pioneer work (1), which led to the recognition of lumiflavin and lumichrome as breakdown products of riboflavin which had been exposed to light. Later, photochemical reactions were used for the preparation of flavin derivatives (2), as well as for the modification of enzyme-bound flavins (3). The photoreactivity of flavins has, surprisingly, never been used as a tool for the specific labeling of active centers. In contrast, an FAD analog modified in the adenine moiety, 8-azidoadenosine-FAD, has been used for labeling studies with D-amino acid oxidase and glucose oxidase (4). In contrast to typical active site-directed reagents, which identify reactive nucleophiles in an enzyme active site, photoaffinity reagents can be used to identify the location of the binding site within the protein, even when such sites do not contain highly reactive nucleophiles. Some of the most effective photoaffinity reagents have proven to be arylazides, which generate highly reactive nitrenes upon irradiation (5). 8-Azidoflavins should fulfill the criteria expected of a good photoaffinity label (5–7). 1) The binding is specific and very tight, so that only a stoichiometric amount of reagent is necessary; this is essential to minimize nonspecific labeling. 2) 8-N_3-flavin has a strong absorption in the visible (λ_{max} ~ 450 nm), so the nitrene can be generated without use of short wavelength light which might damage the protein. 3) In the absence of light, 8-N_3-flavins are stable. We have synthesized flavin coenzymes carrying the azido function at position 8, and describe some of their properties and the labeling of some typical flavoproteins.

MATERIALS AND METHODS

The holoenzyme and apoprotein forms of D-amino acid oxidase from pig kidneys (8, 9), flavodoxin from *Megasphera elsdenii* (10, 11), riboflavin-binding protein from egg white (12), Old Yellow Enzyme from yeast (13, 14), and glucose oxidase from *Aspergillus niger* (15, 16) were obtained as described in the literature cited. Bovine serum albumin was from Armour (Chicago) and bovine pancreatic ribonuclease A (5 × crystallized) from Sigma.

Methylhydrazine sulfate was from Eastman. *O*-Methylhydroxylamine was from Aldrich. Sodium bisulfite was from Mallinckrodt Chemical Works. ATP was from Sigma. Riboflavins were converted to the FAD level using the FAD synthetase system, partially purified from *Brevibacterium ammoniagenes*, as described by Spencer *et al.* (17). 8-F-riboflavin (18) was a generous gift from Drs. S. Kasai and K. Matsui, Osaka City University, Osaka, Japan, or was synthesized according to their procedure (18). Spectroscopic measurements were carried out with a Cary 17, 118, or 219, or a Kontron Uvikon 820 recording spectrophotometer at 25 °C if not specified otherwise. HPLC[1] separations were carried out with a Kontron Anacomp 220 system using a Merck RP-18 analytic column (0.45 × 25 cm) and a gradient of 10–50% methanol, 5 mM ammonium acetate, pH 6. In this system, flavins were eluted in the following order: 8-OH-riboflavin > FAD > 8-(NHOH)-riboflavin > FMN > 8-N_3-riboflavin. The latter two flavins are fairly close to each other in the elution profile. 8-N_3-flavin samples at 0–4 °C were irradiated for the indicated times using a Smith-Victor Sun Gun filtered through the Pyrex wall of a thermostatted water bath. The extent of covalent labeling was estimated by the absorbance at the λ_{max} of the irradiated enzyme following precipitation with trichloroacetic acid, centrifugation, washing with 2 ml of 5–10% trichloroacetic acid, and dissolving in a pH 7.0 solution of 8 M guanidine hydrochloride, and expressing this as a percentage of the initial absorbance of the 8-N_3-flavin enzyme before light. Alternatively in some cases the irradiated enzyme was dialysed *versus* 8 M guanidine hydrochloride or, in the case of D-amino acid oxidase,

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[1] The abbreviation used is: HPLC, high pressure liquid chromatography.

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dialyzed over several days with 0.02 M pyrophosphate, pH 8.5, containing 2 M KBr, conditions known to effect quantitative removal of noncovalently bound flavin (9). It should be emphasized that relating the absorbance of the covalently bound flavin to that of the starting 8-N_3-flavin enzyme is only an approximate (but convenient) way of expressing the extent of labeling, as it assumes that the molar extinction of the product is the same as that of the starting 8-N_3-flavin. As some of the potential products have higher extinction coefficients (see "8-NR-substituted Flavins as Models for Light Reaction Products") the "% covalent labeling" values quoted in this paper will be somewhat higher than the true values, depending on the derivatives formed. Use of ^{14}C-labeled 8-N_3-flavin would be required for more quantitative estimation of the extent of labeling, as well as for identification of which protein residues were attacked in the photolabeling.

Synthesis of 8-Azidoflavins from 8-NH_2-flavins—8-NH_2-flavins (19) (~0.1 mmol) were diazotized as described elsewhere (20) at 0 °C, and the resulting solution (volume 5–10 ml) was treated in the dark with 1 mmol of a saturated solution of NaN_3 at the same temperature for 20 min. The mixture was allowed to warm to ambient temperature for several hours and then cooled again overnight. The precipitate which formed was washed thoroughly with distilled water and then dried under vacuum at 50 °C. Care must be taken that all manipulations are carried out in the absence of light. The 8-N_3-riboflavin obtained by this procedure is an orange-reddish solid which decomposes at $T > 200$ °C. 8-N_3-riboflavin ($C_{16}H_{17}N_7O_6$, $M_r = 403.36$) requires: C, 47.64; H, 4.25; N, 24.31%. Found: C, 48.10; H, 4.33; N, 24.08%. Crystalline 8-N_3-lumiflavin and 3-CH_3,8-N_3-lumiflavin were obtained similarly, starting from the corresponding lumiflavins (19). Both the 8-N_3-riboflavin and 8-N_3-lumiflavin showed the strong typical azide stretch vibration at 2100 cm^{-1} in IR spectra. Both compounds were pure as judged by HPLC analysis in the system described above. On mass spectroscopic analysis, no M^+ peak could be observed because of decomposition of the 8-N_3-flavins. 8-N_3-riboflavin is also formed by reaction of 8-diazoriboflavin with hydoxylamine.

Synthesis of 8-Azido- and Related Flavins from 8-F-flavins—8-N_3-FAD was prepared by reacting 8-F-FAD at 5–10 mM with 30 mM sodium azide overnight in 20 mM sodium pyrophosphate, pH 8.5, at 25 °C, taking care to exclude all light. For the coupling with apo-D-amino acid oxidase, the stock solution was then diluted to 20–25 μM with sodium pyrophosphate, pH 8.5, and a stoichiometric amount of apo-D-amino acid oxidase (9) was added. After 20 min at 25 °C, the reaction mixture was centrifuged to remove any turbidity which developed and cooled to 0–4 °C for irradiation. Details of coupling with other FAD-specific proteins are given in the appropriate section. 8-N_3-FMN was synthesized in the same way from 8-F-FMN and diluted as described above for coupling to apoproteins. In general, all 8-N_3-flavoproteins were subjected to gel filtration on Sephadex G-25 before irradiation in order to minimize non-active site-labeling. 8-(HONH)-riboflavin was prepared by reacting 8-F-riboflavin with 0.5–2 M NH_2OH in the pH range 5–7, following spectral changes to completion of the reaction. At pH >8, the reaction with NH_2OH produces predominantly 8-OH-riboflavin, which was identified by comparison with authentic 8-OH-riboflavin (20) both by HPLC and spectroscopic properties. The mechanism of formation of 8-OH-flavin was not studied further. 8-(CH_3-NH-NH)-riboflavin was prepared by reacting 8-F-riboflavin with 0.2 M CH_3NHNH_2 in 0.1 M sodium phosphate, pH 7.5, at 25 °C. 8-(CH_3-ONH)-riboflavin was prepared by reacting 8-F-riboflavin with 1 M CH_3ONH_2 in 0.1 M sodium phosphate, pH 7.5, at 25 °C. The compounds were purified by HPLC as described above. The extinction coefficients were determined by extrapolation from that for 8-F-riboflavin (11.8 mM^{-1} cm^{-1}) and for the 8-(HONH)- and (CH_3-ONH)-riboflavins, by conversion to 8-NH_2-riboflavin upon reduction with dithionite and reoxidation. The extinction coefficient at 476 nm for 8-NH_2-riboflavin was redetermined for this work to be 42 mM^{-1} cm^{-1} by weight, and 43 mM^{-1} cm^{-1} by titration with riboflavin-binding protein, in good agreement with the value reported by Berezovskii *et al.* (19). We have used the average value of 42.5 mM^{-1} cm^{-1} for calculation purposes. This value is slightly lower than that reported by Kasai *et al.* (18).

RESULTS

Preparation of 8-N_3-flavins and Spectral Properties—The flavin position which is most amenable to introduction of chemical functions is C(8), since it is particularly chemically reactive and its chemistry has been well studied (20, 21, 22). A further possible point of chemical substitution would be C(6) (23). The synthesis of isoalloxazines carrying the azido group at position 8 can be achieved via two routes. 8-Amino-lumiflavin or -riboflavin can be diazotized (19, 20) and the flavin 8-diazotate converted (*in situ* or after isolation) with azide to yield crystalline 8-N_3-flavin. This method yields products of high purity and is suitable for the preparation of large amounts of material. (8-N_3-flavin is formed also upon addition of NH_2OH to the flavin 8-diazotate. This reaction, which has not been investigated in detail, is likely to proceed via addition of NH_2OH to the diazo group followed by elimination of H_2O). The compounds prepared in this way are pure by HPLC analysis, have the expected C,H,N analysis, and possess the strong IR stretching band at 2100 cm^{-1} typical of azides. The method of choice for the preparation of 8-N_3-flavins for enzyme studies is the direct conversion of 8-F-FAD or 8-F-FMN to the corresponding N_3 derivative by reacting at neutral pH with azide. This method has the advantage that light can be excluded rigorously during the preparation. The resulting 8-N_3-flavins have identical spectral properties to those obtained starting from the 8-NH_2-flavins, show the same HPLC profiles, and behave identically on light exposure. The absorption spectrum of neutral 8-N_3-riboflavin in aqueous solution (Fig. 1 and Table I) is closely similar to that of 8-(CH_3O)-flavins (24), and different from that of normal 8-(CH_3)-flavins. This indicates similar effects of the -O-CH_3 and -N_3 groups on the chromophore. The analogy is extended to the spectra of protonated 8-N_3-flavin and of its monoanionic form (Fig. 1). In the case of 8-(CH_3-O)-flavin, the site of protonation has been shown to occur at N(1) (pK ~ 1) and formation of the monoanion results from dissociation of the N(3)-H (pK ~ 9) (24). At high pH, 8-N_3-flavins are not stable; the nature of the decay and of the spectral changes accompanying it have not been investigated further. 8-N_3-flavins exhibit an unusual and very pronounced solvatochromy (Fig. 2). In apolar and aprotic solvents, the single-banded absorption observed in aqueous media is resolved into two well separated bands with maxima in the near UV and around 450 nm. In addition, the band in the blue region is now well resolved. This phenomenon might be ascribed to the effect of

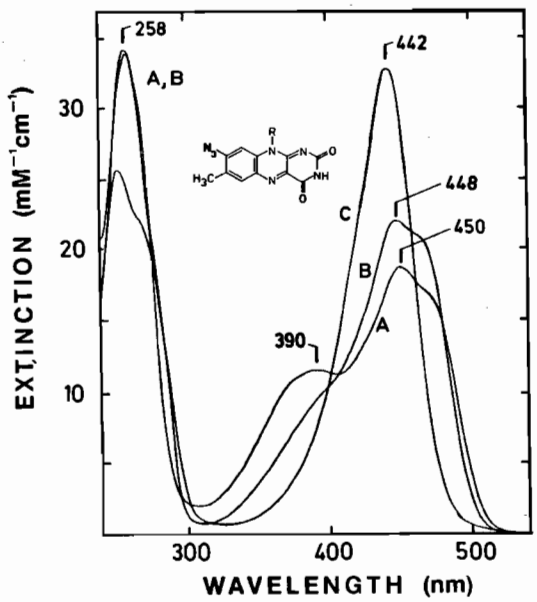
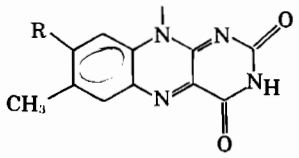


FIG. 1. **Spectra of 8-azidoriboflavin as a function of pH.** *Curve A*, in 0.1 N NaOH; *curve B*, in 0.1 M phosphate buffer, pH 7.0; *curve C*, in 6 N HCl.

TABLE I

Spectral properties of 8-azidoflavins and of related compounds

Structure of substituted flavin[a]	Conditions	Absorption maxima	References or comments
			
		nm	
R = -N_3	pH 7.0	448 (22),[b] 257 (34.4)	
	6 N HCl	442 (33), 259 (34.4)	
	0.1 N NaOH	450 (18.6), 390 (11.6), 252 (25.9)	
	Acetonitrile	470 (15.6), 448 (19.1), 368 (9.5)	
	Benzene	484 (14.3), 455 (18.5), 357 (8.0)	
R = -NH_2	pH 7.0	476 (42.5)	Ref. 16, *cf.* text
R = -NH-NH-CH_3	pH 7.0	489 (29), 256 (43)	
	pH 2.0	444 (18.9), 366 (9.7), 265 (36.8)	
R = -NHOH	pH 7.8	482 (23.5), 346 (11.5)	pK ~ 6
	pH 3.0	474 (31)	
R = -NH-OCH_3	pH 8.0	480 (21.3), 364 (14.4), 254 (29.4)	
	pH 3.4	474 (35.7), 254 (45.9)	pK = 5.9
R = -N_2^+	pH 1.0	475 (7), 324 (15), 262 (38)	
R = -OCH_3	pH 7.0	436 (22.6), 380 sh (7), 262 (27.2)	Ref. 18
R = -OH	pH 7.0	475 (41)	pK = 4.8, Ref. 18

[a] The N(10) substituent is either ribityl (riboflavin) or methyl (lumiflavin). This variation of substitution has, in general, negligible effects on the spectral properties of isoalloxazines.

[b] Values in parentheses are extinction coefficients (millimolar).

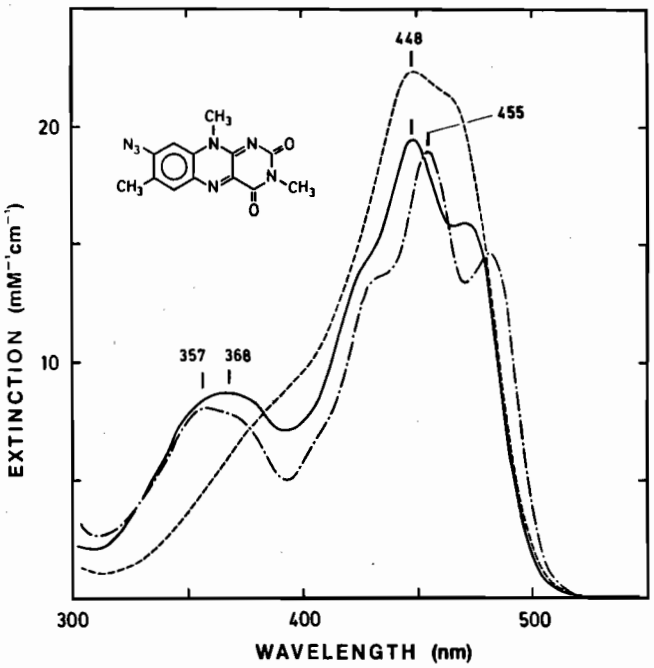


FIG. 2. **Solvatochromy of 8-azidoflavin.** 8-N_3-lumiflavin dissolved in 0.1 M phosphate buffer, pH 7.0 (– – –), in acetonitrile (——), and in 1% acetonitrile, 99% benzene (–·–).

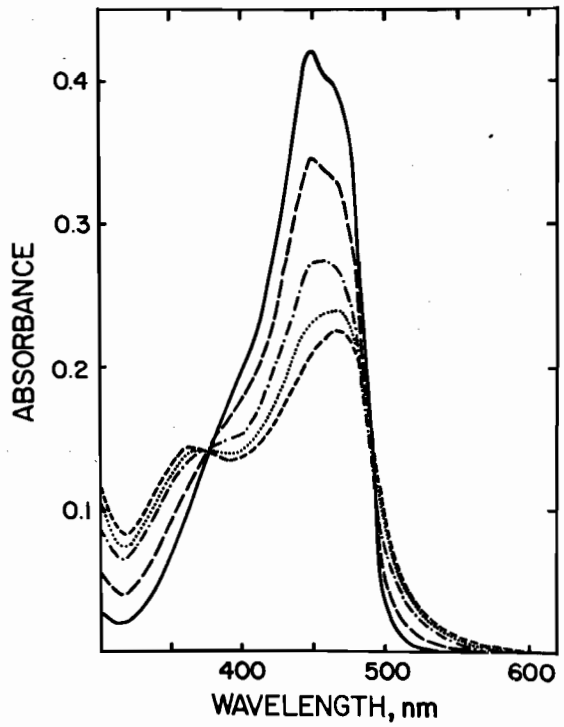


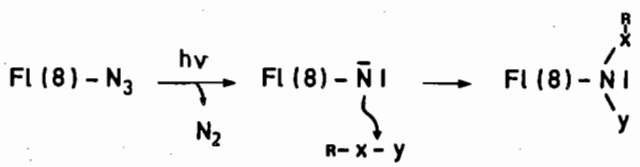
FIG. 3. **Spectral changes during irradiation of 8-N_3-riboflavin.** 8-N_3-riboflavin (20 μM) in 0.1 M sodium phosphate, pH 7.5, at 0–4 °C, was irradiated with white light (~5 × 10^4 ergs cm^{-2} s^{-1}) filtered through a Corning 3-73 glass filter to cut off all light of wavelength less than 420 nm. 8-N_3-riboflavin before irradiation (——), after 85 s of irradiation (– – –), after 175 s (–·–), after 355 s (···), and after 535 s (-----).

the solvent on the electron distribution in the polarized -N_3 substituent, and may be of use in probing the polarity of the flavin environment in enzymes. 8-N_3-flavins are quite fluorescent, although their light sensitivity requires small slit widths in determination of spectra. Examination of 8-N_3-riboflavin showed that it had an excitation spectrum identical with its absorption spectrum, with emission maximum at 515 nm (*cf.* 530 nm for riboflavin and with an intensity approximately 1.5 times that of riboflavin.

Light Reactivity of 8-N_3-flavins—Fig. 3 shows the results of irradiating 8-N_3-riboflavin with very low intensity visible light (~5 × 10^4 ergs cm^{-2} s^{-1}) at neutral pH and 0–4 °C. This demonstrates the extreme light sensitivity of 8-N_3-flavins. The half-life of such a solution exposed to typical room light

is approximately 2 min. For that reason, extreme care must be taken to exclude all light when working with this flavin. The spectral changes which occur upon irradiation consist of a decrease in extinction coefficient, a shift in the λ_{max} from 448 to 470 nm, and increased absorbance at 300–370 nm and 500–600 nm. In fact, the depth of the trough at 310 nm and the ratio of absorbance at 450/520 nm can be used as very sensitive indicators of whether the flavin has been exposed to any light during handling. This photochemical action spectrum has not been determined. While UV light will cause spectral changes such as those shown in Fig. 3, we were more interested in the use of visible light. By use of cutoff filters it was shown that visible irradiation was most efficient in the wavelength region 400–500 nm, as expected from the absorption spectrum. Thus, light of wavelengths less than 320 nm, which might be absorbed by protein, can be filtered out simply by irradiation through the wall of a Pyrex glass thermostatted bath without affecting the generation of the active nitrene from 8-N_3-flavins. The final product consists of several different species. High pressure liquid chromatography (see "Materials and Methods") separates four fractions, two of which have been identified as 8-NH_2-riboflavin and 8-(HONH)-riboflavin, while the others appear to be flavin breakdown products (results not shown).

8-NR-substituted Flavins as Models for Light Reaction Products—The possible insertion reactions of a flavin 8-nitrene at an enzyme active center are summarized in Scheme 1. Depending on the chemical nature of the reacting group, flavins carrying different substituents at N(8) can be expected to be formed. Since these substituents can affect the spectral properties of the chromophore, their systematic study might help identify protein residues involved in the photoreaction. Accordingly, several flavin models were synthesized by nucleophilic displacement of F^- in 8-F-riboflavin (18, 22). The spectra of these are shown in Fig. 4. Reaction of the nitrene with a water molecule should produce 8-(HONH)-riboflavin (Scheme 1, R = H, x = O, y = H), which has a peak at 482 nm in its anionic form (Table I). This flavin has a pK of ~6 losing the long-wavelength tail absorption at low pH, and with an increase in extinction of the band in the visible (Table I). This pK cannot be ascribed to ionization of the 8-NH-OH function (to yield 8-NH-O^-), in agreement with the finding that 8-(NH-O-CH_3)-riboflavin has a pK of 5.9 and shows similar spectral properties in its anionic and neutral forms. That the ionization at pK ~ 6 corresponds to a deprotonation can be deduced from the mobility of the species above and below the pK on HPLC and by comparison with the mobility of anionic flavins (*e.g.* 8-O^--riboflavin) or neutral flavins (*e.g.* riboflavin or 8-NH_2-riboflavin). The position of deprotonation (*i.e.* the tautomeric structure of the neutral form) can be either C(8)-NHOR or N(1)-H (cf. *structures* on Fig. 4). In the ionized state, the negative charge is likely to be strongly delocalized into the aromatic system as shown by the meso-



x = C, O, N, (S)
y = C, H, (O, N)

SCHEME 1. **Possible reaction modes of photochemically generated flavin 8-nitrene with protein residues.**

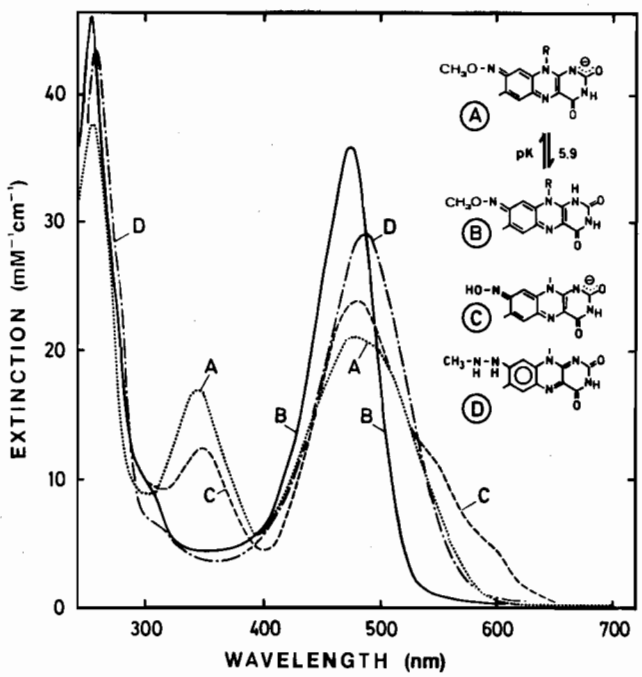


FIG. 4. **Spectral properties of various 8-substituted flavins as model compounds for reaction products resulting from reaction of photochemically generated flavin 8-nitrene with amino acid residues.** *Curves A*, anionic and *B*, neutral forms of 8-(CH_3O-NH)-riboflavin at pH 8.0 (phosphate buffer, 0.1 M) and at pH 3.4 (diluted acetic); *curve C*, spectrum of the anionic form of 8-(HO-NH)-riboflavin in 0.1 M phosphate buffer, pH 7.8; *curve D*, absorption spectrum of 8-(CH_3-NH-NH)-riboflavin in 0.1 M phosphate buffer, pH 7.0. The substituent at N(10) is ribityl.

meric structure (*A*), (Fig. 4) since the spectral characteristics are closely similar to those of (paraquinoid) 8-OH-flavins (24).

The product of the reaction with a serine or threonine hydroxyl was modeled with the 8-(NH-OCH_3)-riboflavin (Scheme 1, R = C, x = O, y = H) (Fig. 4 and Table I). Neither the 8-(NH-OH)- nor the 8-(NH-OCH_3)-riboflavin is appreciably fluorescent, in contrast to 8-NH_2-flavin (19), which has an intense orange fluorescence (emission maximum 535 nm, intensity at pH 7, 20 °C, 4.3 times that of riboflavin). As will be detailed later, upon reduction with dithionite (and reoxidation) of either of these flavins, 8-NH_2-riboflavin is formed. Photoreaction of the 8-N_3-flavin with a lysine ϵ-amino group or with the amino terminus of the protein chain should produce 8-(NH-NH-R)-flavin (Scheme 1, R = C, x = NH, y = H). The model for this was the 8-(NH-NH-CH_3)-riboflavin, which has a single peak at 489 nm (Fig. 4 and Table I). Photoreaction of the 8-N_3-flavins with the C-H bonds of the polypeptide chain should produce an 8-(NH-C)-flavin. The spectra of such riboflavins have previously been reported, for example 8-(NH-C_2H_5)-riboflavin has an extinction coefficient at 493 nm of 45.5 mM^{-1} cm^{-1} (25). Finally, extraction of a "hydride" by the nitrene would produce 8-NH_2-riboflavin, the properties of which are well documented (19).

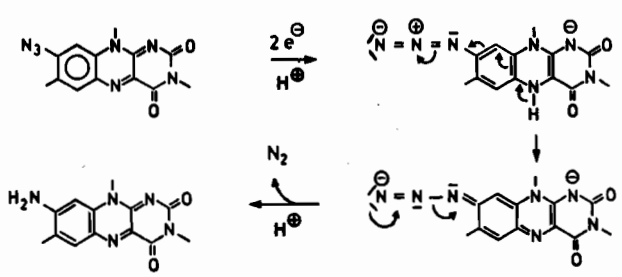
Formation of 8-NH_2-flavins by Elimination of N_2 from Reduced 8-N_3-flavin and of HOR from Reduced 8-(NH-OR)-flavins—When 8-N_3-riboflavin is reduced with dithionite and the products are allowed to reoxidize with air, the absorption spectrum and fluorescence properties of the reoxidized material are those of 8-NH_2-riboflavin. Similarly, when 8-N_3-riboflavin is reduced coulometrically under anaerobic conditions, the first observed spectral change is that from the spectrum of 8-N_3-riboflavin to that of 8-NH_2-riboflavin, fol-

lowed by reduction of 8-NH_2-riboflavin. On reoxidation with air, oxidized 8-NH_2-riboflavin is obtained. While reduction and elimination of N_2 might occur without reduction of the flavin, in analogy to results with other arylazides (6, 26), the elimination of N_2 as a property of reduced 8-N_3-flavin was shown by experiments with 8-N_3-FAD-D-amino acid oxidase and 8-N_3-FAD-glucose oxidase (see below), in which it was shown that substrate reduces the enzyme, followed by the spontaneous generation of oxidized 8-NH_2-FAD enzyme, even under anaerobic conditions. The mechanism of the reaction can be envisaged as shown in Scheme 2. Formation of 8-NH_2-riboflavin was also observed upon reduction of 8-(NH-OH)- and of 8-(NH-OCH_3)-riboflavin and could proceed conceivably by a similar mechanism.

8-N_3-riboflavin is also converted readily to 8-NH_2-riboflavin by reaction with thiols such as dithioerythritol. While the reaction has not been studied in detail, a red-colored intermediate is detectable very quickly on mixing, which then decays to give 8-NH_2-riboflavin in >90% yield. The final product was identified as 8-NH_2-riboflavin by its spectral properties and by HPLC analysis. No reduction of the flavin was observed, and in this case, it appears likely that the reaction proceeds by reaction of the thiol with the azide itself, followed by N_2 elimination, as has been observed with other arylazides (26). In this respect, the reaction is probably formally similar to that found with sulfite (see below).

Reaction of 8-N_3-flavin with Sulfite—The reaction of sulfite with 8-N_3-flavin also generates 8-NH_2-flavin. However, in analogy with the reaction with thiols, a distinct reaction intermediate other than reduced flavin can be observed. Upon the addition of 10 mM sulfite at pH 8.8, 25 °C, a species with a spectrum shifted to the red is formed with a $t_{1/2}$ of ~5 min (Fig. 5). This species then breaks down in a first order reaction with a $t_{1/2}$ of ~120 min to form 8-NH_2-riboflavin as the main product. The identity as 8-NH_2-riboflavin was determined based on absorbance and fluorescence spectra, on spectral changes upon binding to riboflavin-binding protein, and by HPLC. The rate of formation of the intermediate is directly dependent upon the concentration of sulfite, with a second order rate constant of 0.019 M^{-1} min^{-1} at 25 °C, pH 8.5. The rate of breakdown of the intermediate is independent of sulfite concentration, but is temperature-dependent, and is slow enough at 0–4 °C that a pK of 7.6 could be determined by spectral titration. At pH 5.0, the intermediate flavin has a single peak at 473 nm with an extinction coefficient of ~30 mM^{-1} cm^{-1} (Fig. 5); at pH 9.7, it has a maximum at 530 nm with an extinction coefficient of 32 mM^{-1} cm^{-1}. The rate of breakdown of the intermediate is also pH-dependent, with a $t_{1/2}$ of 28 min at pH 5.6, 25 °C.

The properties of the intermediate allow a deduction of the mechanism of this reaction: The high absorbance in the visible spectrum indicates that the flavin chromophore has not undergone reduction, *e.g.* by attack of sulfite at position N(5).



SCHEME 2. **Mechanism of formation of 8-NH_2-flavin upon reduction of 8-N_3-flavin.**

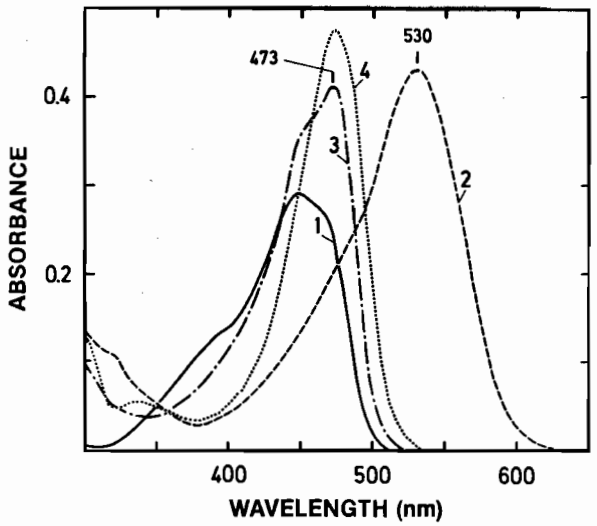
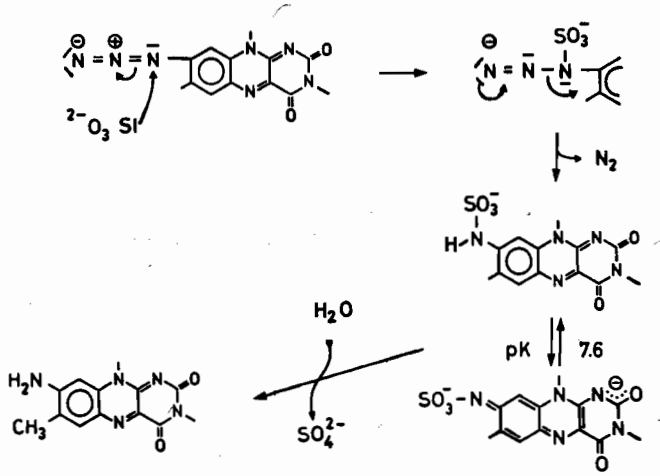


FIG. 5. **Reaction of 8-N_3-riboflavin with sulfite.** *Spectrum 1* (——), 8-N_3-riboflavin in 20 mM sodium pyrophosphate, pH 8.8, 25 °C. Sodium sulfite (10 mM) was added, and *spectrum 2* (– – –) was produced over the course of 35 min. The temperature was lowered to 4 °C and the pH was adjusted to 5 by the addition of HCl to give *spectrum 3* (–·–). The pH was raised to 7.8 by the addition of NaOH, the temperature was raised to 25 °C, and the adduct was allowed to decay to produce *spectrum 4* (····).



SCHEME 3. **Proposed mechanism for the reaction of sulfite with 8-N_3-flavins leading to 8-NH_2-flavins via a flavin 8-sulfonylamide.**

Addition of SO_3^{2-} to the proximal nitrogen of the 8-N_3 group as shown on Scheme 3 is formally a reduction of the azido group and might be expected to result in elimination of N_2, as in the case of reduction of arylazides by thiols (26). The product formed is a sulfuric acid amide of a very electron-deficient aromatic amine, which can be expected to have a pK as low as the observed pK of 7.6. The marked spectral changes accompanying deprotonation are similar to those observed with 8-OH- and 8-SH-flavins, where in the anionic form delocalization to a paraquinoid mesomeric structure occurs (22, 24). The hydrolysis of the sulfonylamide appears to be pH-dependent and leads directly to the 8-NH_2-flavin (Scheme 3).

Labeling of Riboflavin-binding Protein with 8-N_3-riboflavin—Riboflavin-binding protein binds 8-N_3-riboflavin stoichiometrically at neutral pH, with a $K_d \leq 10^{-7}$ M, resulting in increased resolution in the spectrum (Fig. 6). An extinction coefficient of 21,000 M^{-1} cm^{-1} for 8-N_3-riboflavin was determined from titrations with riboflavin-binding protein. This is in good agreement with the value of 22,000 M^{-1} cm^{-1} deter-

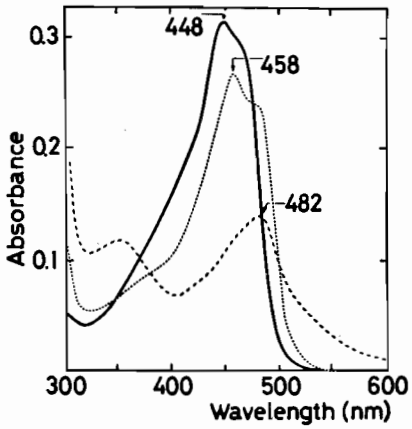


FIG. 6. **Spectral changes during irradiation of 8-N_3-riboflavin bound to riboflavin-binding protein.** 8-N_3-riboflavin (14 mM) in 0.1 M sodium phosphate, pH 7.5, 0–4 °C (——), after addition of 20 μM riboflavin-binding protein (· · · ·), and after 25 min irradiation with white light at an intensity of ~8 × 10^6 ergs cm^{-2} s^{-1} at 0–4 °C (– – –).

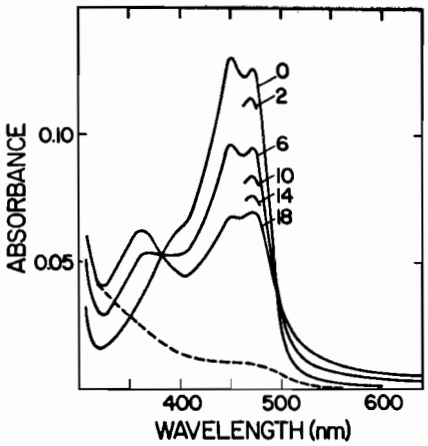


FIG. 7. **Spectral changes on light irradiation of 8-N_3-FMN-flavodoxin.** The substituted protein (1 ml) obtained by gel filtration with Sephadex G-25 equilibrated with 0.05 M phosphate, pH 7.0, 0 °C, was irradiated with white light at an intensity of ~8 × 10^6 ergs cm^{-2} s^{-1} for the times (in minutes) shown. *Dotted line*, spectrum of the 18-min sample after precipitation with 5% trichloroacetic acid and dissolving the precipitate in 1 ml of 8 M guanidine hydrochloride plus 0.1 M phosphate, pH 7.

mined by direct measurement (cf. above and Table I). The results of irradiating 8-N_3-riboflavin/riboflavin-binding protein are shown in Fig. 6. At first sight they are similar to the results with the free riboflavin. The major difference is the striking decrease in the light sensitivity of 8-N_3-riboflavin upon binding to this protein. Even with the order of magnitude increase in light intensity, much longer irradiation times are necessary for the light reaction to occur. In fact, it proved necessary to irradiate at 0–4 °C in order to decrease the rate of dissociation of the flavin from the protein (27). Otherwise, the much greater sensitivity of the free flavin to light and the relatively fast off-rate at 25 °C resulted in the majority of the photoreaction occurring with the free 8-N_3-flavin, and in this case leading to less covalent labeling.

Approximately 21% of the flavin in Fig. 6 is covalently bound to the protein. The noncovalently bound flavin could be quickly removed by passage over a Sephadex G-25 column equilibrated with 8 M guanidine or by dialysis overnight *versus* several changes of 0.1 M sodium acetate, 1 M KBr, pH 4.5, at 0–4 °C. This latter treatment was gentle enough that subsequent dialysis *versus* neutral phosphate buffer produced protein which was still able to bind riboflavin. This was taken advantage of to pass a sample of riboflavin-binding protein through three cycles of labeling followed by dialysis to remove noncovalently bound flavin, in order to increase the amount of covalently bound flavin to approximately 30%. The spectrum of covalently labeled riboflavin-binding protein is similar to that of the light-irradiated sample shown in Fig. 6 and shows a peak at 480 nm which trails out past 600 nm and a second peak at 340 nm. By comparison with the spectra of model compounds (Fig. 4 and Table I), covalent linkage of the flavin to an oxygen function of the protein (serine, threonine, or tyrosine hydroxyls) would be indicated.

Labeling of Flavodoxin with 8-N_3-FMN—8-N_3-FMN is bound tightly by *M. elsdenii* apoflavodoxin, exhibiting the usual marked shifts in absorption spectrum of the flavin on binding (ϵ_{448} decreases from 22,000 to 17,900 M^{-1} cm^{-1}), and the flavin remains associated with the protein on gel filtration on a column of Sephadex G-25. The holoenzyme is poorly photoreactive, as shown in Fig. 7, with spectral changes similar to those found rapidly with free 8-N_3-riboflavin (Fig. 3). On precipitation of the irradiated protein with 5% trichloroacetic acid, conditions known to separate flavin and apoprotein quantitatively (10), only approximately 8% of the flavin absorbance remains associated with the protein. This finding was as expected, since in flavodoxins, according to x-ray crystallographic studies (28) and studied with chemically reactive flavin probes (29), the 8-CH_3 region of the flavin moiety is exposed to solvent. On photoirradiation, the presumed flavin nitrene would be expected to react predominantly with H_2O, as does the nitrene from 8-N_3-flavin in free solution. It should also be noted that photoreaction of 8-N_3-FAD-glutathione reductase does not lead to covalent modification of the protein (30). From x-ray crystallographic studies of the native enzyme it is known that the flavin 8-CH_3 position is also in contact with solvent (31).

Labeling of D-Amino Acid Oxidase with 8-N_3-FAD—8-N_3-FAD binds reasonably tightly to apo-D-amino acid oxidase, but with comparatively little change in absorption spectrum on binding (λ_{max} = 448–450 nm). The resulting holoenzyme can be separated from the free 8-N_3-FAD by gel filtration on Sephadex G-25 and is catalytically competent in an assay monitoring the formation of phenylglyoxylate from phenylglycine (32). However, the activity decreases rapidly indicating progressive inactivation of the enzyme. This behavior is consistent with the observation that 8-N_3-FAD-D-amino acid oxidase is reduced readily by D-alanine under anaerobic conditions, but in the reduced state N_2 is eliminated concomitant with formation of oxidized 8-NH_2-FAD enzyme, which, presumably because of its low oxidation reduction potential (33), is no longer capable of reduction by D-alanine (results not shown).

The spectral changes seen upon irradiation of 8-N_3-FAD enzyme were similar to those seen with riboflavin-binding protein and free 8-N_3-riboflavin, but the decrease in absorbance was less (Fig. 8). The light sensitivity of 8-N_3-FAD-D-amino acid oxidase is similar to that of free 8-N_3-riboflavin, in contrast to when bound to riboflavin-binding protein. The extent of covalent attachment of the FAD to the protein in a large number of experiments, carried out at pH 8.5 or pH 7.3, was in the range of 10–16%, measured either as the amount of flavin remaining with the protein after precipitation with trichloroacetic acid or the amount remaining after dialysis for 3–5 days against 2 M KBr, 20 mM pyrophosphate, pH 8.5, conditions sufficient for making apoprotein (9). The spectrum of 8-N_3-FAD-labeled D-amino acid oxidase from which non-

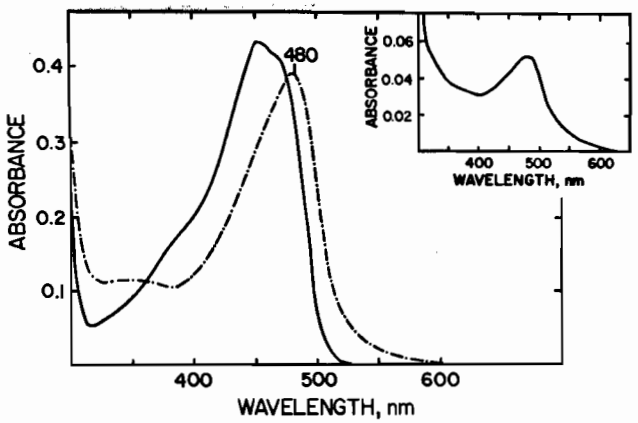


FIG. 8. **Spectral changes on irradiation of D-amino acid oxidase containing 8-N_3-FAD.** Conditions: 20 mM sodium pyrophosphate, pH 8.5, 4 °C. 8-N_3-FAD (32 μM) was incubated with apo-D-amino acid oxidase (47 μM) for 20 min at 25 °C. The sample was then cooled to 4 °C. ——, spectrum of 8-N_3-FAD bound to D-amino acid oxidase; -·-, after irradiation with white light (~8 × 10^6 ergs cm^{-2} s^{-1}) for 8 s. *Inset*, spectrum of covalently linked flavin obtained by dialyzing the irradiated sample for 4 days against 2 M KBr, 20 mM pyrophosphate, pH 8.5, and overnight against 20 mM pyrophosphate, pH 8.5, before taking the spectrum.

covalently attached flavin has been removed by the latter method is shown in Fig. 8. The small extent of covalent attachment obtained is in the range found for nonspecific labeling (see below) and is consistent with previous studies, which indicated that the flavin 8-position is exposed to solvent in D-amino acid oxidase (29). However, surprisingly, the presence of 10^{-3} M benzoate had very little effect on the extent of covalent attachment of the flavin to the protein, even though it did slow down the light reaction approximately ten-fold. This result is unexpected and unexplained since previous studies with 8-Cl-FAD enzyme and 8-mercapto-FAD enzyme indicated that in the presence of benzoate the flavin 8-position was no longer exposed to solvent (29).

Labeling of Old Yellow Enzyme with 8-N_3-FMN—8-N_3-FMN is bound tightly to apo-Old Yellow Enzyme, with pronounced spectral shifts (Fig. 9) typical of those found for binding of all flavins to this protein (14, 34). The resulting holoenzyme is much less sensitive to light than 8-N_3-FMN, and prolonged light exposure was required to generate the changes shown in Fig. 9. The changes are similar to those found with free 8-N_3-flavins. This, coupled with the very low extent of covalent attachment of the flavin to the protein (7%), is consistent with previous studies which indicate that the flavin 8-position is freely exposed to solvent in this protein (29).

Labeling of Glucose Oxidase with 8-N_3-FAD—Previous studies with chemically reactive 8-substituted flavins had indicated that in glucose oxidase the flavin 8-position was shielded from solvent (29). As in the case of riboflavin-binding protein (see above), it was therefore expected that more extensive covalent labeling of 8-N_3-FAD-glucose oxidase would be found on light irradiation than with the other 8-N_3-flavoproteins. This was indeed found to be the case. 8-N_3-FAD binds reasonably tightly to apoglucose oxidase; when equimolar concentrations (~4 × 10^{-5} M) of 8-N_3-FAD and apoglucose oxidase are passed through a Sephadex G-25 equilibrated with 0.1 M phosphate, pH 7.0, approximately 50% of the added flavin elutes with the protein. The resultant holoenzyme is reduced rapidly by glucose, and, as with the corresponding D-amino acid oxidase, conversion to 8-NH_2-FAD takes place. Now, however, the 8-NH_2-FAD enzyme is also reduced by

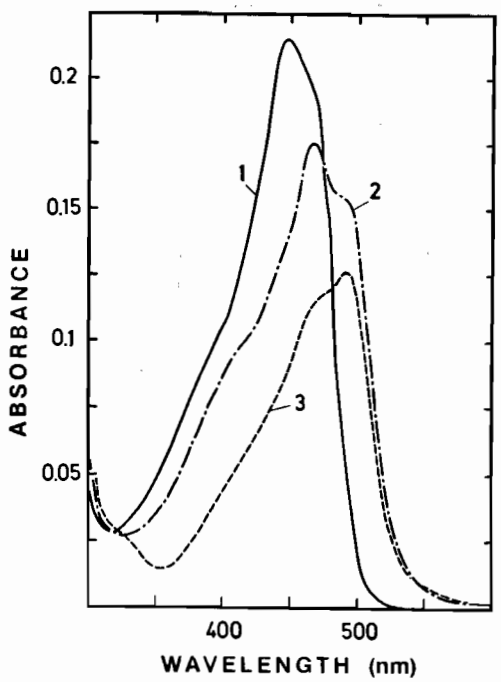


FIG. 9. **Spectral changes associated with binding of 8-azido-FMN to apo-Old Yellow Enzyme and subsequent light irradiation.** *Curve 1*, 8-N_3-FMN (10 μM), in 0.1 M phosphate, pH 8.0, 8 °C; *curve 2*, after addition of 20 μM apo-Old Yellow Enzyme; *curve 3*, after irradiation with white light at an intensity of ~2 × 10^6 ergs cm^{-2} s^{-1} for 8 min.

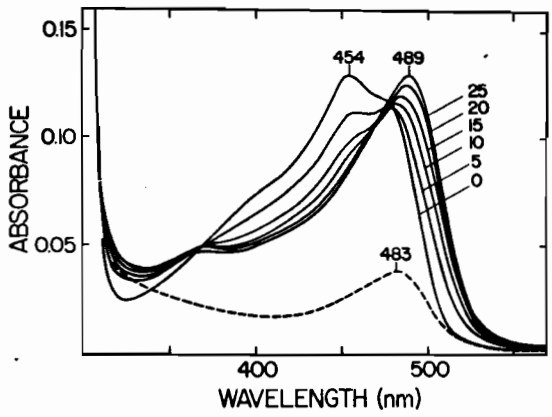


FIG. 10. **Spectral changes on light irradiation of 8-N_3-FAD-glucose oxidase.** The reconstituted protein (1 ml) obtained by gel filtration with Sephadex G-25 equilibrated with 0.1 M phosphate, pH 7.0, 0 °C, was irradiated with white light at an intensity of ~8 × 10^6 ergs cm^{-2} s^{-1}, for the times (in minutes) shown. *Dotted line*, spectrum of the 25-min sample after dialysis for 18 h *versus* 8 M guanidine hydrochloride plus 0.05 M phosphate, pH 7, and volume readjusted to 1.0 ml.

glucose, so that it is only when turnover is complete that the spectrum of the oxidized 8-NH_2-FAD enzyme is found.

The 8-N_3-FAD enzyme is orders of magnitude less sensitive to light than the free flavin. Results of a typical experiment are shown in Fig. 10. At the end of the 25-min irradiation, the sample was dialyzed in the dark for 18 h *versus* 8 M guanidine HCl, and the extent of covalent labeling was estimated as 29% from the spectrum of the dialyzed protein (*dashed line* of Fig. 10). Similar experiments were carried out with different times of irradiation. Covalent labeling of 18% was found after 14 min, 25% after 22 min, and 20% after 34 min. It therefore appears as though secondary breakdown

reactions occur on prolonged irradiation. At the shorter periods of irradiation, it is probable that unreacted 8-N_3-FAD was still present, judged by the appearance of 8-NH_2-FAD on treatment with dithionite.

Control Tests for Nonspecific Labeling—A series of control experiments with bovine serum albumin and bovine pancreatic ribonuclease was carried out with 8-N_3-riboflavin to explore complications which might arise in flavoproteins through nonspecific interactions. These results are summarized in Table II. In all cases the light-induced spectral changes were rapid, as in the case of free 8-N_3-riboflavin. Despite the relatively low concentrations of protein, it is clear that significant covalent attachment of the flavin to the protein may occur, especially at pH 8.5. As would be expected of nonspecific reactions, the extent of covalent labeling is decreased on lowering the concentrations of both reactants. From these results it is clear that in order to avoid nonspecific labeling, irradiation of 8-N_3-flavoproteins should be carried out at pH 7, where feasible, at a concentration of approximately 10^{-5} M, and preferentially after gel filtration or dialysis to remove non-liganded 8-N_3-flavin.

DISCUSSION

The properties of 8-N_3-flavins, particularly those of the coenzyme level corresponding to the naturally occurring FMN and FAD, offer the possibility of their being ideal photoaffinity probes for the active sites of flavoproteins. Thus, when the native flavin is removed and the apoprotein incubated with 8-N_3-FMN or 8-N_3-FAD, specific incorporation of the appropriate coenzyme form is obtained. For example, apoflavodoxin, a protein specific for flavins at the FMN level, binds only 8-N_3-FMN, with a $K_d < 10^{-7}$ M, and does not bind 8-N_3-riboflavin or 8-N_3-FAD. Similarly, riboflavin-binding protein binds only 8-N_3-riboflavin, and the apoproteins of glucose oxidase and D-amino acid oxidase bind only 8-N_3-FAD. That the 8-N_3-flavin is bound stoichiometrically and in the active site is also evident from the fact that the 8-N_3-flavin is reduced by the specific substrate for the particular enzyme. Thus, especially when any excess 8-N_3-flavin is removed by gel filtration, it would be expected that any photolabeling of the protein should occur specifically in the active site region. Both free in solution and bound to protein, 8-N_3-flavins are stable in the absence of light, except in strong alkaline solution. The free 8-N_3-flavins are extremely light-sensitive, especially in aqueous solvent. Under these conditions a predominant product is 8-(NHOH)-flavin, the product expected of reaction of a nitrene with H_2O (35). When potential reductants are present in the system (organic solvent, buffer, or the ribityl side chain of the flavin itself) 8-NH_2-flavin is also formed as a major product. Photolysis of the 8-N_3-flavin bound in the flavoprotein active site would be expected to lead to a variety of products, depending on the environment of the photochemically generated active species (nitrene). If the flavin is bound to the protein in such a way that the 8-position is exposed to the solvent, very little covalent binding of the flavin to the protein would be expected, since the photochemically generated nitrene would be expected to react mainly with solvent molecules. Thus, the small extent of covalent labeling obtained with flavodoxin, Old Yellow Enzyme, and D-amino acid oxidase was as expected, since previous studies with flavins bearing chemically reactive groups had indicated solvent accessibility to the flavin 8-position (29). Among the flavoproteins so far studied, significant photolabeling with 8-N_3-flavins has been found only with riboflavin-binding protein and glucose oxidase. Again, these results are in accord with the previous solvent accessibility studies, which showed that the flavin 8-N_3-position was not accessible to solvent in these proteins (29).

TABLE II

Nonspecific labeling of proteins by 8-N_3-riboflavin

Protein and 8-N_3-riboflavin in 0.05 M pyrophosphate, pH 8.5, or 0.1 M phosphate, pH 7.0, 4 °C, at the indicated concentrations, were irradiated with white light at an intensity of $\sim 8 \times 10^6$ ergs cm^{-2} s^{-1} for 10 s. The extent of covalent labeling was estimated by dissolving the trichloroacetic acid-denatured protein in 8 M guanidine HCl, pH 7.0. Essentially the same results were obtained by exposure of the solutions to room light at 20 °C for 15 min. Under both conditions, the light-induced spectral changes were essentially complete.

Sample	pH	Protein concentration	8-N_3-riboflavin concentration	Covalent labeling
		mg/ml	*μM*	%
Bovine serum albumin	8.5	4	50	13.9
	8.5	1	12.5	6.7
	7.0	4	50	6.2
	7.0	1	12.5	3.1
Ribonuclease	8.5	4	50	16
	7.0	4	50	8.9

The fact that the 8-N_3-flavin is bound specifically and stoichiometrically in the active site of the flavoproteins and the demonstration that nonspecific labeling of proteins such as bovine serum albumin and ribonuclease can occur photochemically in the presence of 8-N_3-riboflavin make inappropriate two of the tests commonly employed for specific photolabeling, *viz.* saturation of the photolabeling and competition with the natural ligand. Thus, if an excess of 8-N_3-flavin over that required to bind in the active site were employed, nonspecific covalent photolabeling would be expected in addition to any active site labeling. In like manner, addition of the native flavin could result in the displacement of the specifically active site-bound 8-N_3-flavin (depending on the kinetics of association and dissociation of the flavin from the enzyme), and lead now to nonspecific covalent labeling. Of course, it should be possible to differentiate between such specific and nonspecific labeling by peptide mapping and sequence studies, but this clearly removes these common criteria as being useful ones with 8-N_3-flavoproteins.

One of the main problems associated with the use of 8-N_3-flavins as active site photoaffinity reagents would appear to be caused by a decrease in the quantum efficiency of generation of the nitrene when the flavin is protein-bound. While the photoreactions of free 8-N_3-flavins are very fast, orders of magnitude more light are required for the photoreactions of some 8-N_3-flavoproteins (*e.g.* riboflavin-binding protein, Old Yellow Enzyme, glucose oxidase). Such a phenomenon can result in the complication that the observed photoreaction may occur to a greater or lesser degree with 8-N_3-flavin which has dissociated from the protein, since in all cases of simple flavoproteins there exists a dynamic equilibrium.

$$E\text{-}Fl_{ox} \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} E + Fl_{ox}$$

In most cases k_{off} appears to be slow, with $t_{1/2}$ values at 0–4 °C of hours/days. However, this is not always the case, and particularly with holoproteins prepared with modified flavins, one must be aware of the possibility that a chemical reaction being observed may be occurring with flavin which has dissociated from the complex. In that case, photolabeling with 8-N_3-flavin, particularly if it is a slow process, may be nonspecific, even though the 8-N_3-flavin was originally located in the enzyme active site. An obvious way to overcome this difficulty would be to increase the intensity of light irradiation

in order to increase the proportion of the reaction proceeding in the active site *versus* the nonspecific reaction with the dissociated flavin.

The fact that in no case more than 30% covalent attachment could be observed deserves some comment. It is possible that 8-N_3-flavins decay upon irradiation without the involvement of solvent, *i.e.* in "unproductive" ways. This could occur by intramolecular reactions and ring expansion reactions as observed with other systems (5). We were unable to identify major products other than 8-NH_2- and 8-(NH-OH)-flavins from the photoreaction of 8-N_3-riboflavin in aqueous medium, although clearly a portion of the photoreaction leads to formation of products other than the two just mentioned. This also suggests that other relaxation modes occur for the photochemically formed nitrene, and that the products might be unstable.

The susceptibility of arylazides to reductive elimination of N_2 and conversion to the corresponding arylamine (26) raises some interesting points with respect to 8-N_3-flavins. On addition of a reducing agent such as dithionite, the flavin chromophore is bleached, and on reoxidation with O_2, the flavin is found to be converted quantitatively to the 8-NH_2-flavin, as judged by absorbance, fluorescence, and HPLC analysis. Such a conversion could, in principle, occur by reduction of the azide linkage and elimination of N_2, as occurs in general, *e.g.* upon reaction of thiols with arylazides (26). While this route is not ruled out in the case of reductants such as dithionite, a reductive elimination via primary reduction of the flavin chromophore, as shown in Scheme 2, can also occur with 8-N_3-flavins. This is shown by the fact that D-amino acids will bring about the conversion with 8-N_3-FAD-D-amino acid oxidase, and glucose with 8-N_3-FAD-glucose oxidase. Neither of these reagents react with 8-N_3-flavins in the absence of the specific protein. Reagents which do appear to bring about the conversion without involving intermediate reduction of the flavin chromophore are thiols such as dithiothreitol. They convert 8-N_3-flavins to the corresponding 8-NH_2-flavin. It should be noted that with the flavoproteins studied, the bound 8-N_3-flavin was quite stable in the dark, even over prolonged periods of storage. It would therefore appear that in none of these cases is a thiol residue of the protein situated close to the flavin 8-position. This finding should be contrasted with experiments in which attempts were made to reconstitute apoglucose oxidase with 4-thio-FAD and where rapid conversion to normal (4-oxo)-FAD was found (36). This conversion appears to be due to a suitably positioned protein thiol residue in the active site, as it could be prevented by prior reaction of the apoenzyme with 5,5′-dithiobis(nitrobenzoic acid) (36). These results, taken together, point to the remarkably specific fashion in which the flavin coenzyme must be inserted into the flavin-binding site of the protein, and add weight to the validity of using chemically modified flavin coenzymes to probe flavoprotein active sites. In summary, 8-N_3-flavins appear to be useful additions to the present list of flavoprotein active site probes, as they can provide information by both their dark and light reactions.

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