Light Product of Photoreactive 6-Azido-FAD Bound to Deflavo-Milk Xanthine Oxidase†

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ABSTRACT: Xanthine oxidase from milk was reconstituted with the photoreactive flavin, 6-azido-FAD. While irradiation of the reconstituted enzyme under anaerobic conditions yielded 6-amino-FAD as a light product, aerobic irradiation resulted in formation of an unknown product, which gave the enzyme almost the same activity as that of the native enzyme. The light product could be extracted from the enzyme without breakdown and was found to be highly fluorescent. Upon treatment with phosphodiesterase, this light product was converted to the FMN form. The absorption spectrum of the FMN form has a peak at 464 nm, a shoulder at 450 nm in the visible region, and two peaks at 260 and 298 nm in the UV. Irradiation of free 6-azido-3-methyllumiflavin in the presence of a saturating concentration of oxygen yielded a light product whose absorbance and fluorescence spectra were very similar to those of the light product extracted from the enzyme, suggesting that the two had undergone some common photochemical change at the same place in the isoalloxazine ring. Analysis of the light product of 6-azido-3-methyllumiflavin with ^{1}H NMR and FAB mass spectrometry suggested its possible structure with a new five-membered ring, C(6)=N—O—CH=C(7), adjacent to the benzene ring of the flavin.

6-Azido-FAD is a useful FAD analogue, having a photoreactive azido group at the carbon(6) of the isoalloxazine ring as a substituent (Ghisla et al., 1986). 6-Azidoflavoproteins were reconstituted from several kinds of apoflavoproteins, including those from flavodoxin, Old Yellow Enzyme, D-amino-acid oxidase, lactate oxidase, and riboflavin binding protein (Massey et al., 1986). Upon irradiation of these reconstituted flavoproetins, the spectral changes observed were different from one another, evidently because of different irradiation products. For example, irradiation of 6-azido-FAD D-amino-acid oxidase brought about covalent binding of 85% of the flavin to the protein molecule. This covalent linkage was unstable and was broken upon denaturation of the enzyme, releasing a flavin light product, which was similar to 6-aminoflavin in its spectrum and in the elution profile of HPLC[1] but proved not to be identical to it (Massey et al., 1986). In other cases, such as 6-azidoriboflavin bound to the aporiboflavin binding protein of hen egg white, a highly fluorescent, noncovalently bound flavin light product was formed. In no case was the structure of the light product determined, but it was clear that different products were formed depending on the protein environment around carbon(6) of the isoalloxazine ring, even when the reaction did not result in covalent binding of the flavin to the protein.

In the present work the artificial enzyme, reconstituted from deflavo-xanthine oxidase and 6-azido-FAD, has been irradiated under aerobic and anaerobic conditions. The flavin light products under both conditions were not covalently bound to the protein. The light product under aerobic conditions was a new FAD derivative, while that under anaerobic conditions proved to be 6-amino-FAD. The properties of the former, including its possible chemical structure, will be presented.

Materials and Methods

6-Azido-FAD was synthesized as reported previously (Ghisla et al., 1986). 6-Azido-3-methyllumiflavin was a gift from Dr. Sandro Ghisla, University of Konstanz. Milk xanthine oxidase was purified from fresh unpasteurized cow's milk by modification of the method reported previously (Massey et al., 1969). The pancreatin digestion step was omitted, and the first step of the folate gel affinity chromatography (Nishino et al., 1981) was used as a final procedure. The purified enzyme contained approximately 65% of its active sites in the functional sulfido form (Massey & Edmondson, 1970) and showed xanthine–O_2 activity of around 510 mol urate min^{-1} (mol subunit)$^{-1}$ under standard assay conditions (see below). The value of 37.8 mM^{-1} cm^{-1} was used as the extinction coefficient of MXO at 450 nm (Massey et al., 1969). Deflavo-xanthine oxidase was prepared by the method of Komai et al. (1969). When the deflavo-enzyme was reconstituted with normal FAD, the xanthine–O_2 activity was 450 mol urate min^{-1} (mol subunit)$^{-1}$.

The method of reconstitution of enzyme from deflavo-MXO and the artificial FAD was the same as that reported previously (Saito et al., 1989). Deflavo-MXO was incubated with a slight excess (around 1.3 molar excess) of artificial FAD in 0.1 M sodium pyrophosphate/HCl buffer, pH 7.5, at 20 °C in the dark for 30 min. The reconstituted enzyme was concentrated by centrifugation with a Centricon 30 membrane filter (Amicon), followed by washing with buffer to remove excess flavin. The extinction coefficients of the reconstituted enzyme and the enzyme-bound flavin were calculated based on that of

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[1] Abbreviations: MXO, milk xanthine oxidase; 6-azido-FAD MXO, enzyme reconstituted from deflavo-milk xanthine oxidase and 6-azido-FAD; 6-amino-FAD MXO, enzyme reconstituted from deflavo-milk xanthine oxidase and 6-amino-FAD; HPLC, high-performance liquid chromatography.

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deflavo-MXO at 550 nm, 11.6 mM^{-1} cm^{-1}.

The 6-azido-FAD bound to the reconstituted enzyme or free 6-azidoflavins was irradiated using a commercially available "Sun Gun" at a light intensity of $\sim 3.5 \times 10^6$ erg cm^{-2} s^{-1}, with the cuvette held in a Pyrex glass bath filled with water at 20 °C. The irradiated enzyme was incubated with 2% SDS in 0.08 M sodium pyrophosphate buffer, pH 7.5, at 25 °C for 10 h in the dark, followed by centrifugation through a Centricon 30 ultrafilter. The resulting filtrate containing the extracted flavin light product was loaded on a Sep-Pak C18 cartridge (Waters). After being washed with water, the yellow modified flavin was eluted with 5% acetonitrile. The solvents were evaporated, and the light product, solubilized in less than 200 μL of water, was purified with an Isco Model 2350 HPLC instrument equipped with a Lichrosorb RP 18 column (Chromanetics, 4.6 × 250 mm, 10 μ). The following linear gradient of methanol was used at a flow rate of 1 mL/min at room temperature: at 0 min (when the sample was loaded), solvent A:solvent B = 100:0; at 6 min, A:B = 100:0; at 56 min A:B = 0:100, where solvent A was 5 mM CH_3COOH/NH_4OH, pH 6.0, and solvent B was 40% methanol in 5 mM CH_3COOH/NH_4OH, pH 6.0. The irradiated products of free 6-azido-FAD were desalted with the Sep-Pak C 18 and separated with HPLC in the same way as described above.

6-Azido-3-methyllumiflavin was irradiated and separated on HPLC as follows: 6-azido-3-methyllumiflavin was dissolved in several hundred milliliters of water (concentration was around 2.5 μM). After equilibration with bubbling oxygen for 10 min at 0 °C, the solution was irradiated (100 mL at a time) in a flask held in a Pyrex glass bath filled with water at 0 °C for 1 min. The irradiated solution was loaded on a Sep-Pak C18 cartridge, and the yellow photoproduct was eluted with 3 mL of acetonitrile. After the acetonitrile was evaporated, the light products were dissolved in less than 200 μL of 30% methanol and loaded on the Lichrosorb RP 18 column. The following linear gradient of methanol was used at a flow rate of 1 mL/min at room temperature: at 0 min (when the sample was loaded), solvent A:solvent B = 65:35; at 4 min, A:B = 65:35; at 54 min, A:B = 45:55; at 84 min, A:B = 0:100, where solvent A was 5 mM CH_3COOH/NH_4OH, pH 6.0, and solvent B was 90% methanol in 5 mM CH_3COOH/NH_4OH, pH 6.0. In preparation of the sample for ^1H NMR, the separation of the light products was performed with an Hitachi 655 A HPLC instrument equipped with a Supelco LC 18 T column (Supelco, 4.6 × 250, 5 μ). The following linear gradient of methanol was used at a flow rate of 1 mL/min at room temperature: at 0 min (when the sample was loaded), solvent A:solvent B = 57:43; at 50 min, A:B = 29:71; at 60 min, A:B = 0:100, where solvent A was water, and solvent B was 70% methanol.

Approximately 75 nmol of the light product of 6-azido-3-methyllumiflavin after purification as described above was dissolved in 500 μL of deuterated chloroform (Isotec, 99.8 atom% D, containing 0.05% tetramethylsilane) and used for the ^1H NMR spectrum, which was recorded using a Bruker AMX 500 instrument operating at 500 MHz at 25 °C. FAB mass spectrometry was performed at the Mass Spectrometry Facility, Chemistry Department, University of Michigan.

Enzyme activity was measured in 1 mL of assay mixture containing 98 mM sodium pyrophosphate/HCl buffer, pH 8.5, 0.29 mM EDTA, and 100 μM xanthine at 25 °C, by following the formation of urate at 295 nm.

Absorption spectra were recorded with Cary 219 or Hitachi 3200 recording spectrophotometers or with a Hewlett-Packard diode array spectrophotometer, at 20 °C, unless otherwise indicated. Fluorescence spectra were recorded with a ratio recording instrument designed and built by Dr. D. P. Ballou and Mr. Gordon Ford, University of Michigan.

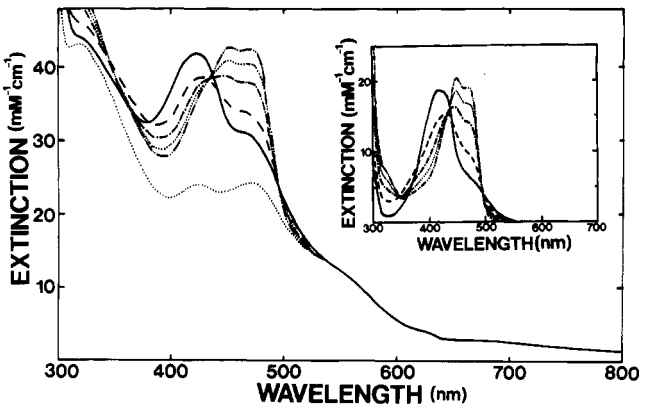


FIGURE 1: Reconstitution and irradiation of 6-azido-FAD MXO. 6-Azido-FAD MXO was reconstituted in 0.1 M sodium pyrophosphate/HCl buffer pH 7.5, then irradiated at 20 °C, as described under Materials and Methods. The spectra before irradiation (—), after irradiation for 10 s (– –), for 15 s (–·–), for 25 s (···, top) and for 50 s (–··–, the end point) are shown. The inset shows the difference spectra between these and the spectrum of the deflavo-MXO (···, bottom).

RESULTS

Reconstitution and Irradiation of 6-Azido-FAD MXO. The spectrum of reconstituted 6-azido-FAD MXO is shown in Figure 1 (solid line). The absorption peak of the reconstituted enzyme and that of the enzyme-bound flavin (obtained as a different spectrum between the reconstituted enzyme and the deflavo-enzyme) was at 422 nm, where the extinction coefficients were calculated to be 41.8 and 18.7 mM^{-1} cm^{-1}, respectively. The latter value was slightly higher than the extinction of free 6-azido-FAD at 428 nm, 17.5 mM^{-1} cm^{-1} (Ghisla et al., 1986). This was consistent with the observation that when deflavo-MXO and 6-azido-FAD were incubated the absorption band of flavin was slightly blue-shifted, and its intensity was also slightly increased, as the reconstitution of enzyme proceeded. The apparent xanthine–O_2 activity of the reconstituted enzyme was >100 mol urate min^{-1} (mol subunit)$^{-1}$ but quickly dropped during turnover, and its accurate determination was difficult. After turnover for 1 min the activity was constant at 32 mol urate min^{-1} (mol subunit)$^{-1}$. As reported previously (Saito & Massey, 1990), upon reduction of 6-azido-FAD MXO with xanthine under anaerobic conditions followed by reoxidation, the absorption spectrum of the enzyme became similar to that of 6-amino-FAD MXO, and the xanthine–O_2 activity of the enzyme also became comparable to that of 6-amino-FAD MXO, 43 mol urate min^{-1} (mol subunit)$^{-1}$. The loss of activity during turnover observed here is probably due to conversion of the enzyme-bound 6-azido-FAD to 6-amino-FAD as a result of reduction of the 6-azido group and elimination of N_2, as observed previously with other 6-azidoflavoproteins (Massey et al., 1986).

The change in the spectrum of 6-azido-FAD MXO upon irradiation is shown in Figure 1. After irradiation for 50 s, the absorption band of the enzyme-bound flavin (obtained as a difference spectrum between irradiated enzyme and deflavo-enzyme) was red-shifted with a new peak at 450 nm, and a clear shoulder at 470 nm (dot-dot-dash line, in the inset). The extinction coefficients were calculated to be 20.5 mM^{-1} cm^{-1} at 450 nm and 18.9 mM^{-1} cm^{-1} at 470 nm. The extinction of the irradiated enzyme at 450 nm was 42.8 mM^{-1} cm^{-1}. The spectra after irradiation for 10, 15, 25, and 50 s

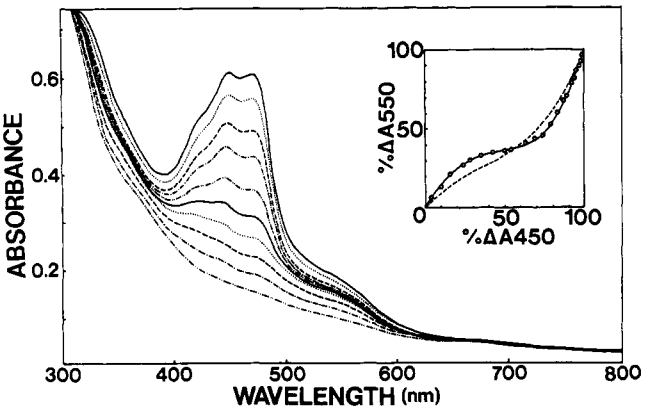


FIGURE 2: Reductive titration of irradiated 6-azido-FAD MXO with sodium dithionite under anaerobic conditions. The irradiated 6-azido-FAD MXO was titrated anaerobically with sodium dithionite in 0.1 M sodium pyrophosphate buffer pH 7.5 at 20 °C. After each addition of dithionite, the absorption spectrum of the enzyme was measured. Only representative spectra are shown for clarity. In the inset the percentage absorbance change at 550 nm is plotted against that at 450 nm. The dashed line represents the same plot obtained in an experiment with native MXO under the same conditions.

showed two isosbestic points at 346 and 434 nm, but the spectrum before irradiation did not pass through these points, suggesting formation of some intermediate compound before the final light product. The xanthine–O_2 activity of the irradiated enzyme was 530 urate min^{-1} (mol subunit)$^{-1}$, comparable to that of the starting native (FAD) enzyme.

The irradiated 6-azido-FAD MXO was anaerobically titrated with sodium dithionite (Figure 2). In the inset of Figure 2, the percent absorbance change at 550 nm (indicating degree of iron–sulfur reduction) is plotted against that at 450 nm (indicating degree of flavin and iron–sulfur reduction) for the irradiated 6-azido-FAD MXO and for native MXO. The two plots are close to each other, suggesting that the order of redox potentials of the chromophores of the irradiated 6-azido-FAD MXO is comparable to that of native enzyme with the flavin light product having a similar redox potential to that of enzyme-bound FAD (Hille et al., 1981; Hille & Massey, 1991).

Extraction of Flavin Light Product from Irradiated 6-Azido-FAD MXO. The flavin was extracted from the irradiated 6-azido-FAD MXO, as described under Materials and Methods. Judging from the absorption spectra of the protein fraction and the low molecular weight fraction, which were separated from each other using Centricon 30 ultrafiltration (see Materials and Methods), 100% of the flavin light product was released from the protein and recovered in the Centricon filtrate (data not shown here). The absorption spectrum of the HPLC-purified flavin light product is shown in Figure 3. The spectrum has a peak at 468 nm, a shoulder at 454 nm in the visible region, and two peaks at 257 and 298 nm in UV region. As shown in the inset, this flavin was eluted as the main peak in the HPLC, but some other minor products were also noted.

Treatment of Extracted Flavin Light Product with Snake Venom. The flavin extracted from irradiated 6-azido-FAD MXO was converted to the FMN form by treatment with *Naja naja* venom, a potent source of phosphodiesterase. As shown in Figure 4, after the venom treatment the absorption peak at 468 nm was blue-shifted at 464 nm, and its intensity was increased by approximately 10%. The peak at 298 nm became much more prominent after removal of the AMP moiety. The fluorescence of the light product after the venom treatment was 11 times higher than that of the original FAD

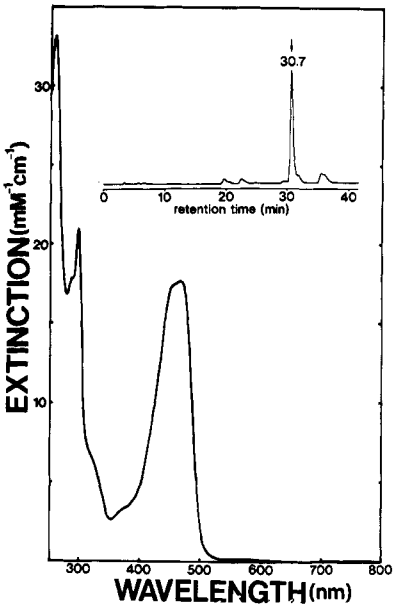


FIGURE 3: Absorption spectrum of flavin light product extracted from irradiated 6-azido-FAD MXO. The flavin light product was extracted from irradiated 6-azido-FAD MXO and purified by HPLC as described under Materials and Methods. The spectrum of the light product contained in the main peak was recorded at 4 °C in the elution solvent of the HPLC (in 5 mM CH_3COOH/NH_4OH at pH 6.0, containing 21% methanol). Above the spectrum the chromatogram of the HPLC monitored at 260 nm is shown, where the main peak is indicated by an arrow, together with its retention time (min).

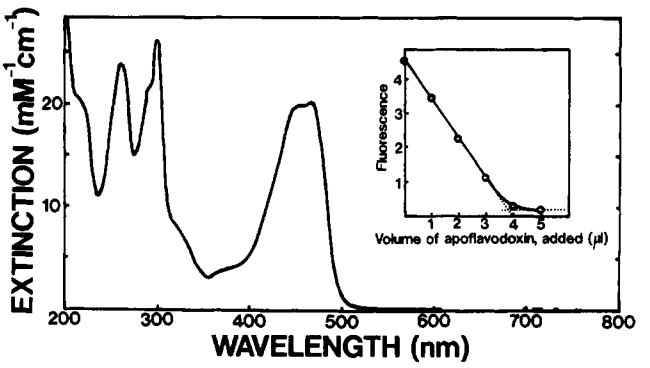


FIGURE 4: Spectrum of FMN form of the flavin light product. The extracted flavin light product (shown in Figure 3) was treated with *Naja naja* venom in 10 mM potassium phosphate buffer, pH 7.0, for 20 min at 25 °C and then loaded on a Sep-Pak C18 cartridge. After washing with water, the flavin was eluted with 5% acetonitrile, and its spectrum was recorded at 4 °C. Inset: The FMN form of light product (A_{464} = 0.026, in 870 μL of 10 mM potassium phosphate buffer, pH 7.0) was titrated with 296 μM apoflavodoxin solution. After each addition of apoflavodoxin, the fluorescence of the flavin (excitation wavelength, 460 nm, and emission wavelength, 510 nm, at 25 °C) was measured. The intensity of the fluorescence (in arbitrary units) is ploteed against the volume of the apoflavodoxin solution added.

form of the light product and 4.8 times higher than that of pure FMN (excitation wavelength at 460 nm and emission wavelength at 510 nm in 10 mM potassium phosphate buffer pH 7.0 at 25 °C). Since the quantum yield of fluorescence of riboflavin has been estimated as 0.25 (Weber, 1961; Moore et al., 1977), it can be calculated that the quantum yield of the FMN form of the light product must be very close to 1.0. To the solution of the FMN form of the light product known concentrations of apoflavodoxin solution were added. As shown in the inset of Figure 4, the strong fluorescence was proportionally quenched by addition of apoflavodoxin, suggesting quantitative binding of the light product to the apoflavodoxin. From the end point of the titration, the extinction

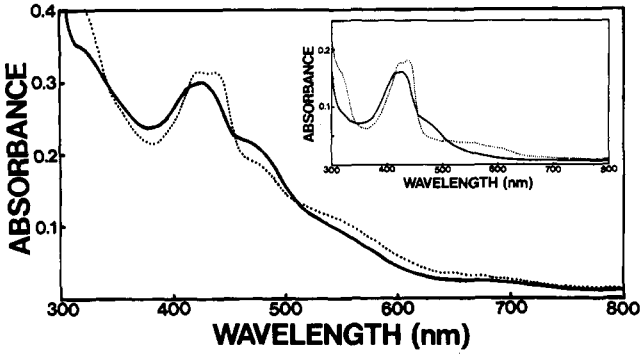


FIGURE 5: Irradiation of 6-azido-FAD MXO under anaerobic conditions. 6-Azido-FAD MXO was reconstituted in 0.1 M sodium pyrophosphate/HCl buffer pH 7.5 (—). The enzyme solution was made anaerobic and irradiated for 20 s (···, end point) at 20 °C. The inset shows the difference spectrum between the reconstituted enzyme and the same concentration of deflavo-MXO before (—) and after (···) irradiation.

coefficient of the light product at 464 nm was estimated to be 20.2 mM^{-1} cm^{-1} for the FMN form and 18.4 mM^{-1} cm^{-1} for the FAD form at 468 nm.

Reconstitution of Enzyme from Fresh Deflavo-MXO and Extracted Flavin Light Product. Xanthine oxidase was reconstituted from fresh deflavo-MXO and the light product extracted from the irradiated 6-azido-FAD MXO (shown in Figure 3). The spectrum of the reconstituted enzyme and that of the enzyme-bound flavin (obtained as a difference spectrum between the reconstituted enzyme and the deflavo-MXO) were quite similar to the corresponding spectra of the irradiated 6-azido-FAD MXO shown in Figure 1. The extinction of the reconstituted enzyme at 450 nm was calculated to be 46.5 mM^{-1} cm^{-1}, and that of the enzyme-bound flavin was 23.9 mM^{-1} cm^{-1}. The xanthine–O_2 activity of the reconstituted enzyme was 580 mol urate min^{-1} (mol subunit)$^{-1}$. All these values are slightly higher than the corresponding values of the irradiated 6-azido-FAD MXO. This is probably because the irradiated 6-azido-FAD MXO contained some minor flavin light products as byproducts in addition to the major one, as evidenced by the HPLC analysis (Figure 3, inset).

From these results it can be concluded that upon irradiation of 6-azido-FAD MXO, the major flavin light product was not covalently bound to the protein molecule and could be extracted from the protein without any chemical conversion.

Irradiation of 6-Azido-FAD MXO under Anaerobic Conditions. 6-Azido-FAD-MXO was irradiated under conditions the same as those in Figure 1 except that the enzyme solution was made anaerobic. As shown in Figure 5 the spectrum of the enzyme after irradiation was different from that shown in Figure 1. The irradiated enzyme and enzyme-bound flavin (obtained as a difference spectrum between the irradiated enzyme and the deflavo-MXO) had an absorption peak at 436 nm, where their extinction coefficients are 43.9 and 21.3 mM^{-1} cm^{-1}, respectively. They also showed a broad absorption band around 600 nm. The xanthine–O_2 activity of the irradiated enzyme was 85 mol urate min^{-1} (mol subunit)$^{-1}$. The presence of a new absorption band centered around 600 nm suggested the formation of 6-NH_2 FAD. As a control study an enzyme was reconstituted from deflavo-MXO and 6-amino-FAD. The resulting enzyme showed an extinction of 43.9 mM^{-1} cm^{-1} at 434 nm, and the contribution of the enzyme-bound flavin to this was calculated to be 19.4 mM^{-1} cm^{-1}. The xanthine–O_2 activity of 6-amino-FAD MXO was 43 mol urate min^{-1} (mol subunit)$^{-1}$. By comparing these results, it was suggested that most of the enzyme-bound 6-azido-FAD was converted to 6-amino-FAD upon irradiation under anaerobic conditions. This was confirmed by HPLC analysis of the flavin light product extracted from the enzyme (data not shown here). The results indicated that oxygen participated in the change of 6-azido-FAD MXO upon aerobic irradiation.

Effect of Oxygen Concentration on Irradiation of Free 6-Azido-FAD. Free 6-azido-FAD in water solution was irradiated after equilibration with argon, air, or 100% O_2. When 6-azido-FAD was irradiated in the presence of oxygen, the absorbance at 470 nm increased to form a shoulder on its absorption spectrum (dotted line in Figure 6A and B), while in the absence of oxygen, it decreased (dotted line in Figure 6C). As the flavin light product in aerobically irradiated 6-azido-FAD MXO had an absorption peak at 468 nm (see Figure 3), the shoulder at 470 nm indicated formation of the same light product. Judging from the height of the shoulder, the yield of the same light product decreased as the concentration of oxygen decreased. This was confirmed by HPLC analysis of the irradiation products (the insets of Figure 6A,B,C). The light product eluted in the peak indicated by the arrow proved to be the same as that extracted from aerobically irradiated 6-azido-FAD MXO in its retention time, absorption spectrum, and fluorescence character (data not

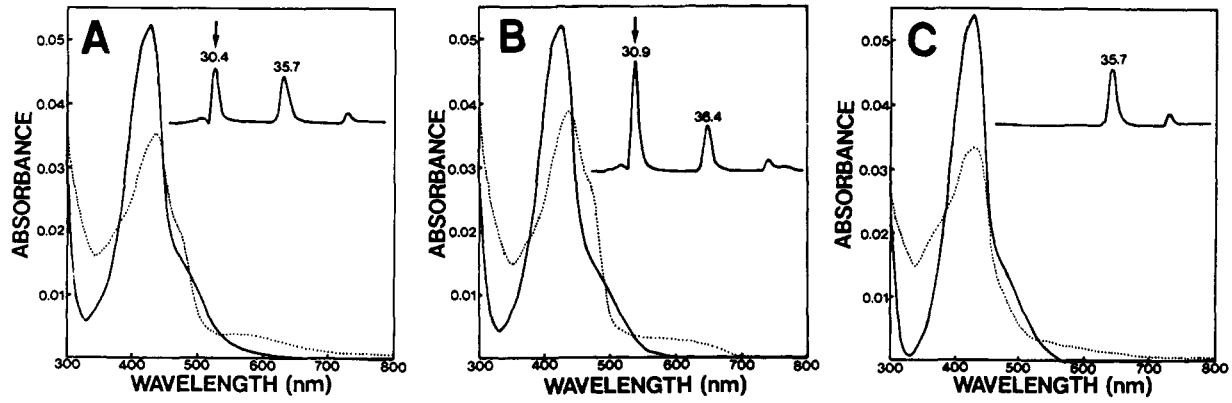


FIGURE 6: Irradiation of 6-azido-FAD under different concentrations of oxygen and separation of the light products by HPLC. 6-Azido-FAD was irradiated in the presence of three different concentrations of oxygen in 0.81 mL of 0.1 M sodium pyrophosphate buffer, pH 7.5, at 20 °C for 150 s. Before irradiation the solution was put in a 1-mL cuvette and equilibrated with air (A), bubbling oxygen (B), or bubbling argon (C) at room temperature for 5 min, then the cuvette was quickly sealed with a silicon cap. Solid and dotted lines represent absorption spectra before and after irradiation, respectively. In each experiment the light products were separated by HPLC as described under Materials and Methods. The linear gradient of methanol was the same as that used in Figure 3A. Part of the chromatogram monitored at 260 nm is shown for each condition. The peak indicated by an arrow contained the light product, which was identical to that extracted from aerobically irradiated 6-azido-FAD MXO. The retention times (min) are shown above each peak.

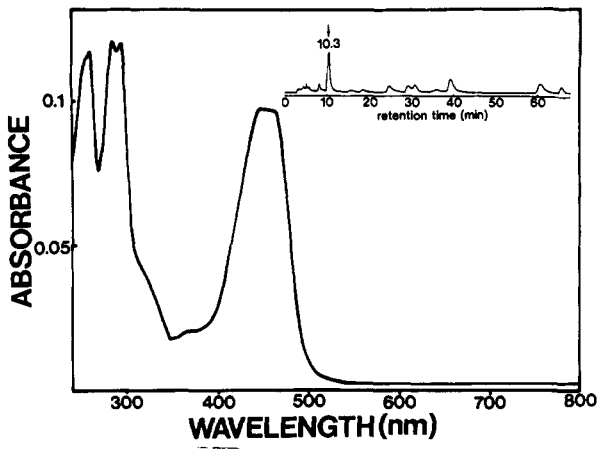


FIGURE 7: Spectrum of the irradiation product of 6-azido-3-methyllumiflavin. An aqueous solution of 6-azido-3-methyllumiflavin was irradiated under equilibration with oxygen at 0 °C, as described under Materials and Methods. The light products were separated by HPLC, and the absorption spectrum of the product in the main peak was recorded in 0.1 M sodium pyrophosphate/HCl buffer, pH 7.5. The inset shows the HPLC chromatogram monitored at 260 nm, where the main peak is indicated by an arrow, and its retention time (min) shown above the peak.

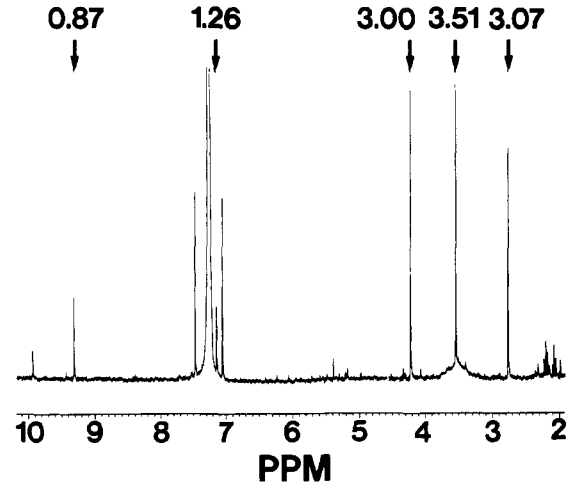


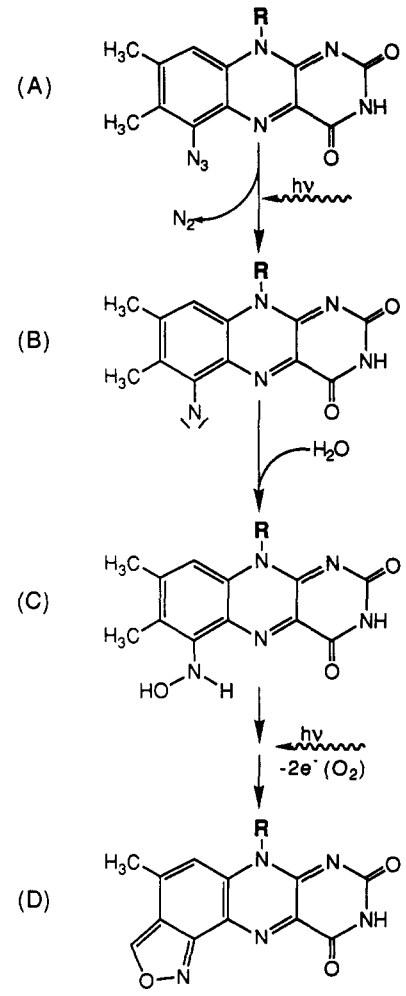
FIGURE 8: ^{1}H NMR spectrum of the light product of 6-azido-3-methyllumiflavin. The irradiation product of 6-azido-3-methyllumiflavin (shown in Figure 7) was analyzed by ^{1}H NMR as described under Materials and Methods. The peaks which were derived from the light product are indicated by arrows. The value above each peak indicates its relative intensity. The Peaks at 7.04, 7.26, and 7.46 ppm were derived from the solvent chloroform.

shown). This peak was clearly larger in Figure 6B (100% oxygen) than in Figure 6A (21% oxygen), and no such peak was observed in Figure 6C (anaerobic).

Irradiation of 6-Azido-3-methyllumiflavin under Saturation with Oxygen. To aid in the structural determination of the light product, we decided to isolate the analogous compound from the model flavin, 6-azido-3-methyllumiflavin. 6-Azido-3-methyllumiflavin was irradiated in solution equilibrated with 100% oxygen at 0 °C. The irradiation products were separated by HPLC as described under Materials and Methods. Figure 7 shows the absorption spectrum of a light product eluted in the peak indicated by the arrow (inset). This light product was very similar to the FMN form of the light product extracted from aerobically irradiated 6-azido-FAD MXO in its absorption spectrum and fluorescence character (not shown here). These two light products can therefore reasonably be expected to have undergone the same photochemical change in the same part of the isoalloxazine ring.

^{1}H NMR Study of the Irradiation Product of 6-Azido-3-methyllumiflavin. Figure 8 shows the ^{1}H NMR spectrum of the light product of 6-azido-methyllumiflavin. As a reference the ^{1}H NMR spectrum of 6-azido-3-methyllumiflavin was taken in deuterated chloroform and by comparison with previous NMR studies of flavins (e.g., Ghisla et al., 1986) the ascriptions within the parentheses could be made as follows: 2.32 ppm (s, 3H, 7-CH_3); 2.52 ppm (s, 3H, 8-CH_3); 3.51 and 4.07 ppm (2 × s, 2 × 3H, 3-CH_3 and 10-CH_3); 7.12 ppm (s, 9-H). Judging from their relative intensities, only the peaks shown in Figure 8 at 2.75, 3.53, 4.21, 7.13, and 9.31 ppm (indicated by arrows) were ascribed to the light product. All these were singlet peaks. Only three peaks showed relative intensities around 3, those at 2.75, 3.53, and 4.21 ppm, and were ascribed to three methyl groups. By comparison with the spectrum of 6-azido-3-methyllumiflavin, it appears that either the C(7)–CH_3 or C(8)–CH_3 was converted to some other structure. The appearance of a peak at 9.31 ppm with relative intensity near 1 indicated a new proton in the light product that was not present in the original 6-azidoflavin. The peak at 7.13 ppm with relative intensity around 1 suggested that the aromatic proton at C(9) of the original flavin was intact. A possible structure of the light product compatible with these NMR data is shown in Scheme I, in which the peak

Scheme I



at 9.31 ppm is ascribed to the proton of the newly formed five-membered ring (see the final product D in Scheme I). This is supported by the fact that a proton on the five-membered ring of anthranil (benzisoxazole) gives a signal at 9.12 ppm (Pouchert & Campbell, 1974). The relative intensities of the peaks at 5.38 and 9.93 ppm were below 0.35, and an-

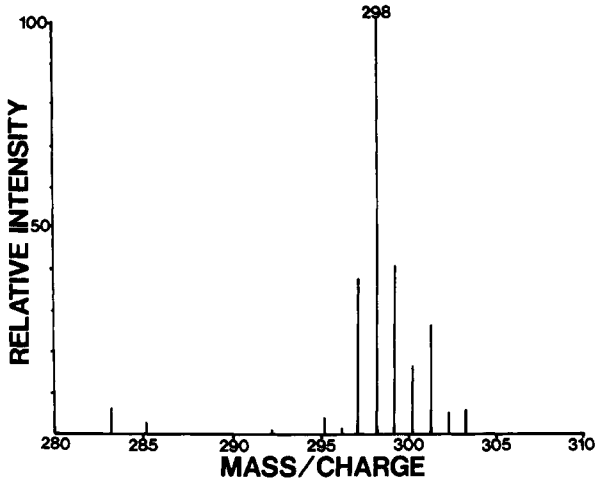


FIGURE 9: Positive-ion FAB mass spectrum of the irradiation product of 6-azido-3-methyllumiflavin. The irradiation product of 6-azido-3-methyllumiflavin (shown in Figure 7), prepared as described under Materials and Methods, was analyzed by positive-ion FAB mass spectrometry. The spectrum shown was obtained by subtracting the blank spectrum (dithiothreitol and dimethylformamide) from the sample spectrum.

other ^{1}H NMR study using a different lot of the same light product in chloroform-*d* did not shown these peaks at all. The intensities of minor peaks around 2.2 ppm relative to those of the peaks indicated by the arrows decreased as the purity of the sample was improved. All these peaks were therefore considered to be derived from some minor contaminants.

FAD Mass Spectrometry of Flavin Light Products. The light product of 6-azido-3-methyllumiflavin was analyzed by positive-ion FAB mass spectrometry. As shown in Figure 9, a molecular ion peak with mass/charge ratio of 298 was detected. This indicated that the molecular weight of the light product was 297, which is consistent with the possible structure proposed above.

The FAD form of the light product extracted from aerobically irradiated 6-azido-FAD MXO was analyzed by negative-ion FAB mass spectrometry. Our sample gave a molecular ion peak at mass/charge ratio of 811 (data not shown here), which is compatible with the FAD derivative having the same five-membered ring as proposed above (*MW* 812).

Redox Potential of Flavin Light Product. The redox potential of the light product from 6-azido-3-methyllumiflavin was determined at 25 °C in 0.1 M phosphate, pH 7.0, by equilibration under anaerobic conditions with redox dyes of known potential, employing xanthine and xanthine oxidase as a controlled source of reducing equivalents (Massey, 1991). The light product was almost completely reduced before reduction of benzyl viologen (E_m, 7 = −359 mV) was evident, and reduction of 1-hydroxyphenazine (E_m, 7 = −172 mV) was complete before reduction of the light product occurred, setting the potential of the flavin between these limits. Rapid equilibration with ideal Nernstian behavior was found with phenosafranin (E_m, 7 = −252 mV) and safranin T (E_m, 7 = −289 mV). The redox potential of the light product was estimated as −276 mV by equilibration with phenosafranin and −280 mV by equilibration with safranin T.

Discussion

Determination of the redox potential of the free flavin light product (from 6-azido-3-methyllumiflavin) shows that the potential is more negative than that of normal flavin (E_m, 7 of FAD = −209 mV; Massey, 1991) by 69 mV. However, the results of anaerobic titration of the irradiated 6-azido-FAD MXO suggest that the redox potential of the flavin light product bound to the enzyme is similar to that of FAD (Figure 2). Furthermore, the irradiated 6-azido-FAD MXO shows an activity comparable to that of the native enzyme, suggesting that the enzyme-bound light product must have a redox potential similar to that of enzyme-bound FAD (Hille et al., 1981; Saito et al., 1987; Hille & Massey, 1991). Thus the enzyme must perturb significantly the potential of the flavin light product, binding more tightly the reduced form in preference to the oxidized form.

The data from ^{1}H NMR and FAB mass spectrometry presented here strongly suggest the structure of the light product extracted from aerobically irradiated 6-azido-FAD MXO to be that shown as the final product D in Scheme I. As reported previously, the substituent at carbon(6) of the isoalloxazine ring is more reactive with solvent-borne reagents in xanthine oxidase than in xanthine dehydrogenase, as shown by experiments using both forms of enzyme reconstituted with various 6-substituted FAD derivatives (Massey et al., 1989). Thus the substituent at carbon(6) is considered to be accessible to solvent in xanthine oxidase. Based on this information, the structure most expected to be formed upon irradiation of 6-azido-FAD (A in Scheme I) bound to the oxidase protein is 6-hydroxylamino-FAD (C in Scheme I), i.e., the product of insertion of the nitrene (B in Scheme I) to water (Ghisla et al., 1986; Abramovitch & Kyba, 1971; Reiser & Wagner, 1971). By a mechanism which has not yet been determined, but which is probably complex, involving both oxygen and light, the flavin C(6)–NHOH reacts with the C(7)–CH_3 to form the five-membered ring of the presumed structure (D in Scheme I). In the formation of the five-membered ring some amino acid residue(s) of the enzyme might play a role in appropriate positioning of the C(6)–NH(OH), which can rotate freely with its C–N bond as an axis. Under anaerobic conditions, the 6-hydroxylamino-FAD may be reduced to form 6-amino-FAD. If this is the case, the possible (photo) reductant is some nearby amino acid residue.

We have also reconstituted deflavo-xanthine dehydrogenase with 6-azido-FAD and studied its photoirradiation. As expected from previous studies indicating a different active site environment of the flavin (Massey et al., 1989; Saito et al., 1989; Saito & Massey, 1991; Hunt & Massey, 1991), the properties of the irradiated dehydrogenase are quite different from those presented here. We are still working on the structural determination of the flavin light product from this enzyme.

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Fragment E-2 from Fibrin Substantially Enhances Pro-urokinase-Induced Glu-Plasminogen Activation. A Kinetic Study Using the Plasmin-Resistant Mutant Pro-urokinase Ala-158-rpro-UK†

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ABSTRACT: In a previous study, it was shown that fibrin fragment E-2 selectively promotes the activation of plasminogen by pro-urokinase (pro-UK) [Liu, J., & Gurewich, V. (1991) *J. Clin. Invest. 88*, 2012–2017]. In this study, the kinetics of this promotion by fragment E-2 was studied. Alanine-158-rpro-UK (A-pro-UK), a recombinant plasmin-resistant mutant, was used in order to avoid interference by UK generation during the reaction. In some experiments, pro-UK was substituted in order to validate the mutant as a surrogate. In the presence of a range of concentrations (0–20 μM) of fragment E-2, a linear promotion of the catalytic efficiency of A-pro-UK against native Glu-plasminogen was seen which was 245.5-fold at the highest concentration of fragment E-2 and 450-fold at the highest ratio of E-2/plasminogen used. The promotion was largely a function of an increase in k_{cat}, since fragment E-2 induced a <10-fold reduction in K_M (8.50–1.40 μM). In contrast to this ligand, ϵ-aminocaproic acid (EACA) induced a biphasic promotion of the activation of Glu-plasminogen which was only 18-fold at maximum. Fragment E-2 did not promote the activation of Lys-plasminogen, but the catalytic efficiency of A-pro-UK was 19.7-fold greater against the open Lys-form than against the closed Glu- form of plasminogen. Fragment E-2 had no effect on the amidolytic activity of A-pro-UK or pro-UK, suggesting that the promotion of their activities was indirect and related to a fragment E-2-induced conformational change in Glu-plasminogen. Since EACA or Lys-plasminogen had relatively little effect on plasminogen activation by A-pro-UK, the fragment E-2-induced conformational change is believed to be novel and different from the well-established open Lys- form of plasminogen. Fragment E-2 caused little (2-fold) promotion of UK-induced plasminogen activation. The observed fibrin fragment E-2 effect on the activity of pro-UK may explain its fibrin specificity and illustrates a mechanism of fibrin-dependent plasminogen activation which is unrelated to fibrin affinity of the activator.

Pro-urokinase (pro-UK)[1] or single chain urokinase-type plasminogen activator is a precursor of two-chain urokinase (UK) which, unlike the latter, induces fibrin-specific clot lysis in a plasma milieu (Gurewich et al., 1984; Zamarron et al., 1984). In plasma, pro-UK is inert and stable, but when a fibrin clot is introduced, plasminogen activation is triggered (Pannell & Gurewich, 1986). Since pro-UK has no significant fibrin affinity, the mechanism by which its fibrin specificity may be explained has remained elusive.

The fibrin dependence of pro-UK has been postulated to be due either to neutralization by fibrin of a competitive in-

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[1] Abbreviations: pro-UK, pro-urokinase or single-chain urokinase type plasminogen activator; UK, two-chain urokinase; fragment E-2, a purified derivative of fibrin; A-pro-UK, alanine-158 mutant recombinant pro-urokinase made by site-directed mutagenesis (Lys-158 → Ala-158); EACA, ϵ-aminocaproic acid; SK, streptokinase; u-PA, urokinase-type plasminogen activator; Plg, plasminogen; Pln, plasmin; pNA, *p*-nitroalanine; F, promotional factor; CNBr-2, cyanogen bromide-2 fragments derived from fibrinogen; t-PA, tissue-plasminogen activator.

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