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Azotobacter Free-Radical Flavoprotein. Preparation and Properties of the Apoprotein*

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ABSTRACT: Precipitation with 3% trichloroacetic acid easily separates an apoprotein from a flavin mononucleotide containing flavoprotein isolated from *Azotobacter vinelandii*. When dissolved in 5% $NaHCO_3$ and stored at 3–4° the apoprotein is stable for flavin mononucleotide binding for at least 11 months. Combination of the apoprotein with flavin mononucleotide is a second-order reaction ($k = 7.9 \times 10^5$ M^{-1} sec^{-1}, pH 5.0, 30°) which proceeds more rapidly near pH 5.0 than at other pH values. Oxidized or reduced reconstituted flavoprotein has an absorption spectrum identical with that of oxidized or reduced unresolved flavoprotein. Reconstituted flavoprotein exhibits electron paramagnetic resonance when reduced with $Na_2S_2O_4$. Urea (1.65 M) inhibits flavin mononucleotide binding by apoprotein but subsequent addition of 0.16 M NaCl relieves this inhibition. Nitration of 4.1 of the 5 tyrosines/27,000 g of apoprotein eliminated *ca.* 90% of its capacity for flavin mononucleotide binding. Flavin bound to the protein protected two of these tyrosines from nitration. The association constants of the apoprotein at 30° with flavin mononucleotide, flavin–adenine dinucleotide, and riboflavin are 7.3×10^8 M^{-1} (pH 5.2), 9.1×10^5 M^{-1} (pH 7.0), and 6.4×10^5 M^{-1} (pH 5.0), respectively. As a working hypothesis these data are tentatively interpreted to mean that the flavin mononucleotide binding site of the apoprotein contains a binding site for phosphate and one binding site, probably containing two tyrosines, for the nonphosphate portion of flavin mononucleotide.

Although apoproteins from several flavoproteins have been prepared (De Luca *et al.*, 1956; Dixon and Kleppe, 1965; Mahler, 1954; Miyake *et al.*, 1965; Negelein and Brömel, 1939; Prosky *et al.*, 1964) an apoprotein has not been prepared from a flavoprotein which under aerobic conditions and neutral pH values forms a stable free radical. Recently, I have prepared such an apoprotein from the unusual flavoprotein of *Azotobacter vindlandii* (Hinkson and Bulen, 1967; Shetna *et al.*, 1964, 1966). The holoprotein reconstituted from the apoprotein and FMN[1] has the same properties as the original, including the ability to form a free radical. In addition to facilitating studies of flavin–protein interactions with respect to flavin binding, this particular apoprotein will allow studies of protein–flavin interactions responsible for free-radical stabilization. In contrast to many apoproteins from flavin-containing proteins (De Luca *et al.*, 1956; Mahler, 1954; Miyake *et al.*, 1965; Negelein and Brömel, 1939), this apoprotein is stable for several months and is easily prepared from a readily obtainable flavoprotein.

Experimental Procedure

Absorption spectra were recorded with a Cary Model 14 spectrophotometer. Spectrophotometric measurements at a single wavelength were determined with a Beckman Model DU or a Gilford Model 240 spectrophotometer. The electron paramagnetic resonance spectrum of a sample was obtained with a Varian Model 4500 spectrometer. A Branson Sonic Power 20-kc sonic oscillator equipped with the S-125 converter was used for sonic oscillation.

Riboflavin was White Label material from Eastman Organic Chemicals, Rochester, N. Y. Sigma Chemical Co., St. Louis, Mo., furnished the FMN (commercial grade) and FAD (grade III). Dr. G. Tollin of this department supplied the recrystallized lumiflavin. Tetranitromethane was a product of Aldrich Chemical Co., Inc., Milwaukee, Wis. All other chemicals were reagent grade obtained from common commercial sources. Except for growth media, deionized distilled water was used throughout.

Protein was measured either by the method of Gornall *et al.* (1949), by the method of Lowry *et al.* (1951), or from A_{280} as calibrated with the method of Lowry

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: TNM, tetranitromethane.

et al. (1951). Flavin concentrations were calculated from the absorbance of the solutions at the appropriate wavelength. Millimolar absorptivities used for these calculations were: FMN, 12.2 at 445 nm (Cerletti, 1959) or 11.1 at 445 nm in 3% trichloroacetic acid (experimentally determined); riboflavin, 12.2 at 450 nm (Whitby, 1953); FAD, 11.3 at 450 nm (Whitby, 1953); 3-nitrotyrosine in peptide linkage, 3.5 at 420 nm (Line *et al.*, 1967).

Fluorometric measurements of the total binding of flavin to protein were made with a G. K. Turner Model 111 fluorometer equipped with a temperature-stabilized door and a recorder. This instrument was thermostated at 30°. The primary filter was Turner No. 110-811 and the secondary filter was Turner No. 110-822. For studies concerned with the rate of FMN binding, an Aminco-Bowman spectrophotofluorometer (excitation wavelength, 450 nm; emission wavelength, 520 nm) equipped with a recorder was employed.

Binding of flavin to apoprotein was determined by measuring the quenching of flavin fluorescence caused by interaction with the apoprotein. Addition of microliter quantities of apoprotein to the reaction mixture initiated reactions in which either the rate of fluorescence change or the total fluorescence change was measured. The molarity of apoprotein in terms of FMN binding sites was routinely determined by titration of apoprotein with FMN as measured by fluorescence quenching of the FMN. Where necessary, corrections were made for small quantities of FMN decomposition products, the fluorescence of which was not quenched by excess apoprotein. This quantity never exceeded 10% of the total FMN present. The volume of each microliter pipet used for these studies was determined by a spectrophotometric technique using concentrated solutions of 2,6-dichlorophenolindophenol.

Growth of *Azotobacter* and flavoprotein isolation were accomplished as previously described (Bulen *et al.*, 1964, 1965; Hinkson and Bulen, 1967) except that for some preparations, sonic oscillation was used to rupture the cells instead of a French pressure cell. No precautions were taken to maintain anaerobicity during protein preparation. Flavoprotein thus prepared migrates in two barely separated bands during low-voltage electrophoresis on cellulose acetate strips but migrates as a single band when the protein is reduced with mercaptoethanol (D. E. Edmondson, personal communication). The latter observation and the nondetection of an amino-terminal amino acid in these preparations (D. E. Edmondson, personal communication) suggest that aerobically prepared flavoprotein is homogeneous just as has been shown for the anaerobically prepared flavoprotein (Hinkson and Bulen, 1967). The two-band and single-band migration during electrophoresis may result from a possible monomer–dimer relationship of the flavoprotein (W. Orme-Johnson and H. Beinert, personal communication).

Estimations of flavoprotein concentrations were made at 358 nm assuming a millimolar absorptivity of 8.82 or assuming a milligram per milliliter absorptivity of 0.282 (Hinkson and Bulen, 1967).

Nitration of the protein with tetranitromethane, a reagent which reacts with only tyrosines and mercapto groups (Riordan *et al.*, 1966), was accomplished in the following manner. In 2.0 ml, 1.9 mg of apoprotein (equivalent to 0.07 μmole of FMN binding sites) in 5% $NaHCO_3$ or 0.05 μmole of holoprotein in 4.4% $NaHCO_3$ was incubated with 5.2 μl of 0.5% (v/v) TNM in 95% ethanol (2.2 μmoles of TNM) for 45 min at 30°. Excess glycyltyrosine was added to quench the reaction. The nitrated protein was freed from all other products of the reaction by passage through a Bio-Gel P-2 column equilibrated with 5% $NaHCO_3$.

Results

Preparation of Apoprotein. Purified *Azotobacter* flavoprotein (Hinkson and Bulen, 1967) was the starting material for preparation of the apoprotein. All operations were performed near 0°. Solutions of flavoprotein (≤2 mg/ml, 0.025 M phosphate, pH 7.0) were adjusted to a final concentration of 3% (w/v) trichloroacetic acid with cold 30% (w/v) trichloroacetic acid. Concentrations of flavoprotein in excess of 2 mg/ml resulted in incomplete removal of FMN, necessitated an extra wash with trichloroacetic acid, and decreased recovery of apoprotein (see Table I). The resulting white to pale yellow precipitate is sedimented at 10,000 rpm (10 min, 0°) and the supernatant is saved as the source of FMN routinely used in these studies. Immediately after preparation, this FMN is 99% pure as judged by its ability to recombine with the apoprotein. Gradually, this FMN decomposes to form another compound (see below). As judged by paper chromatography (Hinkson and Bulen, 1967) small quantities of a fluorescent impurity

TABLE I: Recovery of Apoprotein from *Azotobacter* Flavoprotein.[a]

Sample	Total Protein (mg)	% Recov of Protein
Preparation 1[b]		
Holoprotein	5.0	100
Apoprotein (washed once with trichloroacetic acid)	3.7	74
Preparation 2[c]		
Holoprotein	20.0	100
Apoprotein (washed twice with trichloroacetic acid)	11.5	58

[a] Apoprotein was prepared as described in the text. [b] Preparation 1 contained approximately 2 mg/ml in the starting material. [c] Preparation 2 contained 6.0 mg/ml in the starting material.

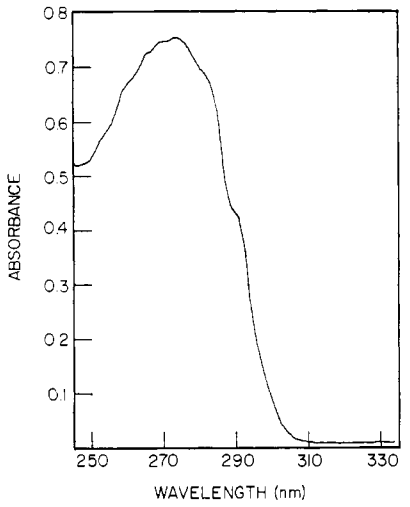

FIGURE 1: Spectrum of apoprotein in 5% $NaHCO_3$. The solution contained 0.54 mg of apoprotein/ml.

were present but only one yellow spot exhibiting fluorescence could be detected. Following a wash with 3% (w/v) trichloroacetic acid, the protein is dissolved in 5% (w/v) $NaHCO_3$ (pH 9.1). These apoprotein solutions, centrifuged to remove small amounts of denatured protein, are stored in a refrigerator. The apoprotein appears to be homogeneous since mercaptoethanol reduced apoprotein migrates as a single band during low-voltage electrophoresis on cellulose acetate strips and since no amino-terminal amino acids could be detected (D. E. Edmondson, personal communication). Nonreduced apoprotein migrates as two barely separated bands during low-voltage electrophoresis. Quenching of FMN fluorescence by one such preparation was undiminished after 11-months storage. Preparations stored in either 0.2 M K_2HPO_4 (pH 9.5) or 0.045 M phosphate (pH 7.0) were *not* stable for this period of time.

The spectrum of the apoprotein (see Figure 1) in 5% $NaHCO_3$ has an absorption peak at 273 nm and absorption shoulders at 253, 260, 265, 270, 283, and 290 nm. Regardless of whether protein concentrations were determined by the biuret method (Gornall *et al.*, 1949) or by the Lowry method (Lowry *et al.*, 1951) the A_{280} for a solution containing 1 mg/ml of protein was essentially the same, 1.34 and 1.30, respectively.

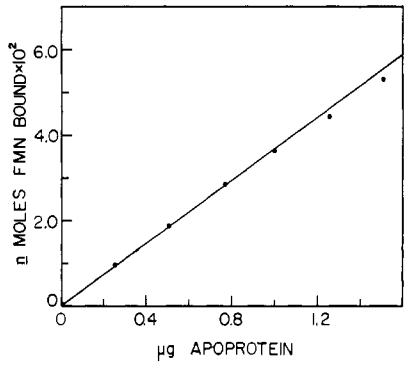

FIGURE 2: Binding of FMN by apoprotein. To 1.0 ml of 0.1 M sodium acetate (pH 5.0) containing 7.9×10^{-8} M FMN were added 0.66-μl aliquots (0.25 μg) of apoprotein. The combining weight of apoprotein per mole of FMN was calculated from this plot to be 27,000 g. The sensitivity of the instrument used for measuring fluorescence was such that 8×10^{-7} M FMN caused an increase in fluorescence of 83 units.

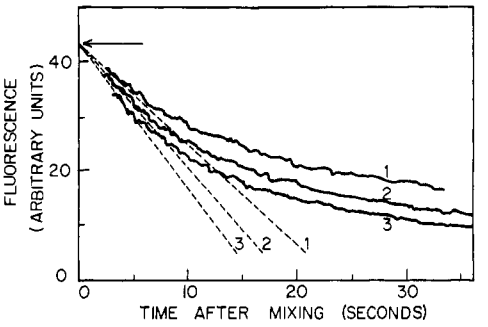

FIGURE 3: Representative plots used to calculate the initial rates of FMN binding. Reaction mixtures (2 ml), thermostated at 30°, initially contained 0.1 M sodium acetate (pH 5.0) and 4.8×10^{-8} M FMN. The reaction was initiated by addition of apoprotein. The solid curves represent traces of fluorescence quenching of FMN by apoprotein at different apoprotein concentrations: curve 1, apoprotein equivalent to 5.6×10^{-8} M FMN binding sites; curve 2, apoprotein equivalent to 7.3×10^{-8} M FMN binding sites; curve 3, apoprotein equivalent to 8.8×10^{-8} M FMN binding sites. The three dashed lines represent the tangents of the solid line curves extrapolated to zero time. From the slopes of these tangents the following initial rates of FMN binding were calculated: line 1, 2.2×10^{-9} M sec^{-1}; line 2, 2.7×10^{-9} M sec^{-1}; line 3, 3.1×10^{-9} M sec^{-1}. The arrow points to the fluorescence of the FMN before addition of apoprotein.

Recombination of Apoprotein with Flavins. With FMN freshly prepared from the holoprotein, over 99% of the FMN fluorescence was quenched by excess apoprotein. From this observation and from the stoichiometry of FMN binding to apoprotein, measured by the fluorescence quenching of FMN with apoprotein (see Figure 2 and results below), it is seen that fluorescence of FMN bound to the apoprotein is negligible. Solutions of FMN gradually decompose to yield a fluorescent product, probably lumiflavin or lumichrome (Hemmerich *et al.*, 1965), whose fluorescence is not quenched by excess apoprotein. During this decomposition neither the A_{445} nor the fluorescence of the solution is diminished and no change of the ability of apoprotein to quench the fluorescence of the remaining FMN is observed. No fluorescence quenching of *bona fide* lumiflavin by the apoprotein occurred. In a mixture containing a 1.8 molar ratio of lumiflavin to FMN, apoprotein-induced fluorescence quenching of the FMN was equal to that observed in the absence of lumiflavin. Although lumiflavin did not affect FMN binding by the apoprotein no experiments were performed with FMN containing more than 10% of this decomposition product.

As calculated from the linear portion of the curve obtained from the titration of FMN with apoprotein (see Figure 2) 1 mole of FMN combines with 27,000 g of apoprotein. Values for this combining weight from other experiments vary from 26,000 to 30,000 g per mole of FMN bound and are comparable with the minimal molecular weight of 31,200 g $mole^{-1}$ determined by Hink-

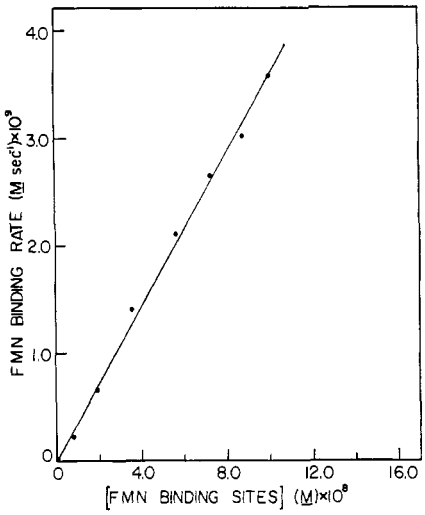

FIGURE 4: Effect of protein concentration (FMN binding sites) upon the initial rate of FMN binding. The reaction mixture, thermostated at 30°, initially contained in 2.0 ml: 0.1 M sodium acetate (pH 5.0) and 4.8 × 10^{-8} M FMN. The reaction was initiated by addition of 0.66–25.0-μl aliquots of apoprotein to give the final concentrations of FMN binding sites indicated. Each point indicates the average initial rate from at least duplicate experiments except for the point at 8.8 × 10^{-8} M FMN binding sites which represents a single determination. The observed values were within 15% of the average value. Recorded plots of fluorescence quenching *vs.* time were extrapolated to zero time and initial rates were calculated from the slopes of these plots at zero time. See Figure 3 for representative plots. The mixing time for these experiments was 3 sec or less. In the instrument used, 4.8 × 10^{-8} M FMN produced 43.5 fluorescence units. The second-order rate constant, k_1, calculated from the slope of the above plot (3.7 × 10^{-2} sec^{-1} = k_1[FMN]) equals 7.7 × 10^5 M sec^{-1}.

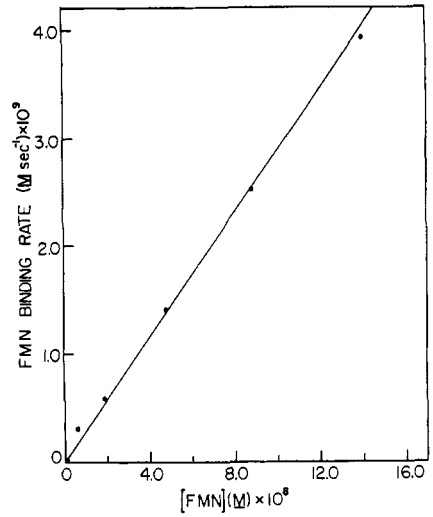

FIGURE 5: Effect of FMN concentration upon the initial rate of FMN binding. The reaction mixture initially contained in 2.0 ml: 0.1 M sodium acetate (pH 5.0) and FMN as indicated. The reaction was initiated by addition of apoprotein equivalent to a final concentration of 3.5 × 10^{-8} M FMN binding sites. See Figures 3 and 4 for other experimental details. The second-order rate constant, k_1, calculated from the slope of this line (2.7 × 10^{-2} sec^{-1} = k_1[apoprotein]) equals 8.1 × 10^5 M sec^{-1}.

son and Bulen (1967).[2] Because this stoichiometry existed, apoprotein concentrations in terms of FMN binding sites were routinely determined by titration of FMN with the apoprotein.

Evaluation of the rate constant for binding of flavin to apoprotein was simplified by calculating the rate constant from the slopes obtained by plotting the initial velocity of FMN binding (see Figure 3 for representative plots) as a function of either initial FMN or initial apoprotein concentrations.[3] This procedure also minimized possible complications which may arise from allowing the reaction to go to completion. As expected for a second-order reaction, the initial rate of binding varies linearly with the initial concentration of either apoprotein or FMN (see Figures 4 and 5). The respective second-order rate constants evaluated from the slopes of these two plots are 7.7 and 8.1 × 10^5 M^{-1} sec^{-1}.

The spectrum of the FMN-reconstituted flavoprotein (Figure 6) has absorption maxima at 452 and 372 nm with a prominent shoulder at 472 nm. After $Na_2S_2O_4$ reduction of recombined holoprotein rather broad maxima at 580 and 614 nm, peaks at 352 and 380 nm, and an isobestic point at 358 nm appear. The ratio A_{452}/A_{372} is 1.19 for the reconstituted flavoprotein and 1.20 for

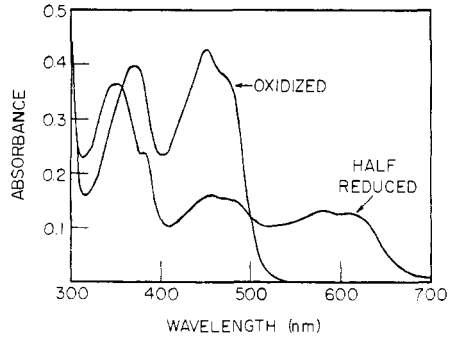

FIGURE 6: Spectrum of reconstituted flavoprotein. Incubation of the apoprotein with FMN proceeded for about 30 min at room temperature (*ca.* 22°) before the spectrum was obtained. In a total volume of 2.1 ml the incubation mixture contained: 5.4 × 10^{-5} M FMN binding sites of apoprotein, 3.7 × 10^{-5} M FMN, 0.17 M $NaHCO_3$, 0.17 M trichloroacetic acid, and enough Tris to adjust the pH to 6.9. Addition of a small quantity of $Na_2S_2O_4$ was used to reduce the flavoprotein.

[2] Drs. W. Orme-Johnson and H. Beinert, University of Wisconsin, have communicated results of their experiments which suggest that the lower value for the combining weight is more nearly correct.

[3] The following is the basis for this simplification. Assume FMN binding to apoprotein occurs according to eq 1, where A

$$A + F \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H \qquad (1)$$

= apoprotein; F = flavin; and H = holoprotein. Then

$$\text{rate} = \frac{-d[F]}{dt} = k_1[A][F] - k_{-1}[H] \qquad (2)$$

Initially [H] = 0 and the change in [A] or [F] is negligible. Therefore, a plot of rate *vs.* [A] or [F] should be linear and the value of k_1, the second-order rate constant, may be calculated from the respective slopes of these plots and the known initial concentrations of either F or A.

TABLE II: Association Constants[a] of Apoprotein with Flavins.

Flavin	Buffer (M)	pH	K_{assn} (M^{-1} at 30°)
FMN	Acetate (0.1)	5.2	7.3×10^{8} [b]
FAD	Phosphate (0.025)	7.0	9.1×10^{5} [c]
Riboflavin	Acetate (0.1)	5.0	6.4×10^{5} [d]
	Phosphate (0.025)	7.0	6.4×10^{5} [e]
	Tris (0.1)	8.1	5.4×10^{5}

[a] These constants were determined at 30° by adding microliter aliquots of apoprotein to flavin solutions. Free-flavin concentrations were calculated from the fluorescence remaining after the system reached equilibrium. The concentration of bound flavin, determined from the difference between the total and free-flavin concentrations, was assumed to represent the concentration of reconstituted holoprotein. Association constants were calculated from the relationship K_{assn} = [holoprotein]/[flavin][apoprotein]. Reaction mixtures used for determination of the indicated constants typically contained the following concentrations: K_{assn} for FMN, 1.0×10^{-7} M FMN and 4.5×10^{-8} M apoprotein; K_{assn} for riboflavin, 1.0×10^{-7} M riboflavin and 4.5×10^{-7} M apoprotein; and K_{assn} for FAD, 4.5×10^{-7} M FAD and 4.5×10^{-7} M apoprotein. Apoprotein molarities refer to the molarity of equivalent FMN binding sites. [b] Average of five determinations ranging from 4.7 to 10.0×10^{8} M^{-1}. [c] Average of four determinations ranging from 5.6 to 17.8×10^{5} M^{-1}. [d] Average of two determinations (6.43 and 6.39×10^{5} M^{-1}). [e] Average of five determinations ranging from 5.4 to 8.5×10^{5} M^{-1}. The same values were obtained in 0.025 M phosphate (pH 7.0) containing 0.4 M NaCl.

the original flavoprotein (Hinkson and Bulen, 1967) Reconstituted flavoprotein reduced with $Na_2S_2O_4$ also exhibits electron paramagnetic resonance at a g value of 2.00. All of these properties are identical with those of the original flavoprotein (Hinkson and Bulen, 1967).

Although FMN is the prosthetic group of the original flavoprotein (Hinkson and Bulen, 1967) the apoprotein quenches the fluorescence of two other common flavins, riboflavin and FAD, as well as that of authenic FMN. However, FMN is bound much more strongly than the other two flavins (see Table II). The apoprotein did not quench the fluorescence of lumiflavin. Apoprotein used for these studies was homogeneous with respect to the protein but not with respect to the forms of the protein present (see above). It is possible that the association constants reported in Table II are not true constants for a given form of the apoprotein. Some preliminary experiments have suggested that the association constants do not vary with the form of the protein present but definitive results have not yet been obtained.

As seen from Figure 7, at 30° the maximal pH for binding of FMN is near pH 5.0. Exposure to pH 3.6 for a day completely inactivates the apoprotein for FMN binding. Riboflavin binding at pH 5.2, 7.0, or 8.0 was too rapid to detect differences in binding rates if, indeed, any differences exist.

Low concentrations of urea practically eliminate binding of FMN by apoprotein (Table III). Addition of NaCl reverses this effect of urea until about 90% of the ability to bind FMN at the given concentration of FMN returns.

TABLE III: Effects of Urea and NaCl upon FMN Binding.[a]

Additions to Buffer (M)	FMN Binding (moles/mole of apoprotein)
None	1.00
NaCl (0.4)	1.00
Urea (1.65)	0.06
+ NaCl (0.08)	0.70
+ NaCl (0.16)	0.89
+ NaCl (0.34)	0.89

[a] For these studies a total volume of 2.5 ml contained 2.7×10^{-7} M FMN and apoprotein equivalent to 1.0×10^{-7} FMN binding sites. The control reaction (no additions) was 0.025 M with respect to phosphate (pH 7.0) and all other reactions were 0.021 M with respect o phosphate (pH 7.0).

Effect of Nitration and Sulfhydryl Reagents upon FMN Binding. Addition of solid *p*-chloromercuriphenylsulfonate or of 5,5′-dithiobis(2-nitrobenzoic acid) to the apoprotein did not affect its ability to quench FMN fluoresence.

Nitration of 4.1 tyrosines/27,000 g of apoprotein reduces its FMN binding ability about 90% (see Table IV). Two of these tyrosines are protected from nitration in the intact flavoprotein. During nitration of in-

TABLE IV: The Effect of Nitration of Apoprotein Tyrosines upon FMN Binding.[a]

Preparation	Nitrotyrosine (moles/27,000 g)	Remaining FMN Binding Sites (moles/27,000 g)
Nitrated apoprotein	4.1	0.11
Apoprotein from nitrated holoprotein	1.8	0.60

[a] See Figure 7 and Experimental Procedure for details of preparation.

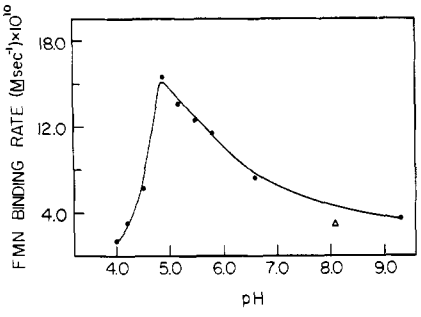

FIGURE 7: The initial rate of FMN binding as a function of pH. The reaction mixture (2.0 ml) contained: 0.1 M sodium acetate, pH after completion of reaction as indicated; 4.7×10^{-8} M FMN; 3.5×10^{-8} M FMN binding sites from apoprotein. The points represent the average of two determinations which was within 5% of the extreme values obtained. The temperature for all reactions was 30°. The triangular point represents the rate in 0.1 M Tris (pH 8.1). Initial rates were calculated as for Figure 3.

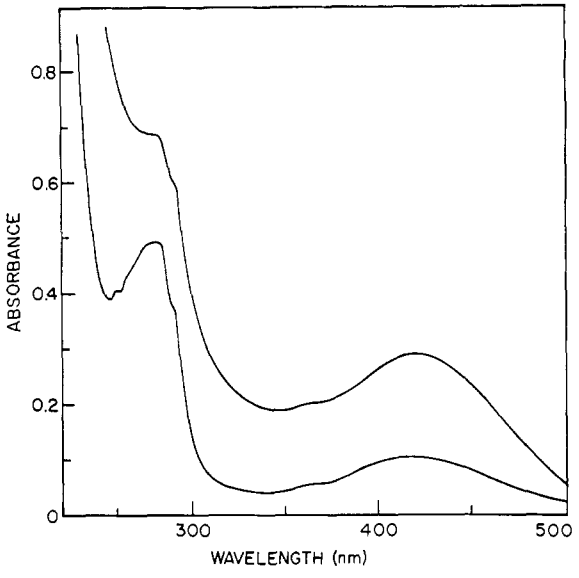

FIGURE 8: Spectrum of nitrated apoproteins in 5% (w/v) $NaHCO_3$. Upper curve, nitrated apoprotein; lower curve, apoprotein prepared from nitrated holoprotein. The material used for the lower curve was obtained by the following method. After passage through a Bio-Gel P-2 column equilibrated with 5% $NaHCO_3$ the fractions containing the nitrated holoprotein were pooled and adjusted to pH 7.0 with glacial acetic acid. To 3.0 ml of this solution 2.0 ml of cold 30% trichloroacetic acid was added which liberated CO_2 from HCO_3^- and precipitated the apoprotein. After washing the apoprotein precipitate once with 3% (w/v) trichloroacetic acid this apoprotein was dissolved in 5% (w/v) $NaHCO_3$. Details of nitration are described in Experimental Procedure.

tact holoprotein, less than 1% of the FMN became fluorescent. Nevertheless, apoprotein prepared from nitrated holoprotein apparently has lost about 40% of the FMN binding sites. In a control experiment, a treatment of apoprotein identical with that employed for preparation of apoprotein from the nitrated holoprotein did not appreciably affect its FMN binding ability (combining weight per mole of FMN, 27,000 g before the treatment and 25,500 g after the treatment).

As suggested by the slight absorption at 380 nm of nitrated proteins (see Figure 8) a small amount of FMN may still be bound. Judged by fluorescence of the apoprotein in 1.8 M acetic acid, a procedure which normally causes fluorescence of the holoprotein (J. W. Hinkson, unpublished observations; Hinkson and Bulen, 1967), this amounted to less than 1% of the original FMN.

Discussion

The ease with which the apoprotein of *Azotobacter* flavoprotein may be prepared, its stability for flavin binding, and the identity of the FMN-reconstituted holoprotein to the original flavoprotein make this apoprotein a valuable tool for studies of flavin–protein interactions. Use of this particular apoprotein for these studies is especially attractive since elucidation of these interactions may shed light upon the mechanism of free-radical stabilization in flavoproteins.

Many properties of the apoprotein from *Azotobacter* flavoprotein are similar to those elucidated by Theorell and Nygaard (1954a,b; Nygaard and Theorell, 1955) for the apoprotein from the FMN-containing "Old Yellow Enzyme." Thus, the values for k_1 are of the same order of magnitude (10^5 M^{-1} sec^{-1}), the values for the K_{assn} for FMN, FAD and riboflavin are similar, and tyrosines are implicated as being involved in flavin binding. The major difference between the two preparations is the pH profile for the rate of FMN binding. Apoprotein from Old Yellow Enzyme bound FMN at a maximal rate near pH 9.0 and at about 60% of the optimal rate between pH 7 and 8. Only a broad pH optimum between pH 6.5 and 8 remained when the reaction was studied in 0.4 M NaCl. In contrast, the rate of FMN binding to apoprotein from *Azotobacter* flavoprotein is optimal at pH 5.0 (see Figure 6) and remains optimal at this pH value even in the presence of 0.4 M NaCl (J. W. Hinkson, unpublished observations).

The sharp decrease in the rate of FMN binding by *Azotobacter* apoprotein at pH values below pH 5.0 suggests that the apoprotein denatures below pH 5.0. However, the ability of the apoprotein to withstand precipitation with trichloroacetic acid (pH ~1) does not seem to be consistent with this possibility. Further investigation is needed before this decrease in rate may be fully understood. At any rate, at the optimal pH for the rate of binding (pH 5.0) only one of the two phosphate hydroxyl groups of FMN would be ionized (Cerletti, 1959). Whether or not the apparent pK of approximately 7.0 suggested by the shape of the descending limb on the alkaline side of the pH optimum results from ionization of a proton from the second hydroxyl group of the FMN phosphate cannot presently be ascertained.

The protection of two tyrosines from nitration by bound flavin may be explained in at least two ways: bound flavin either directly covers these tyrosines or induces changes in protein structure which prevent nitration of tyrosines far removed from the flavin binding site.[4] Complexes between riboflavin and hydroxylated

[4] The second possibility was suggested by Dr. J. A. Rupley of this department during discussions with him about this work.

aromatic compounds exist (Fleischman and Tollin, 1965) so direct involvement of protein tyrosines with FMN binding is theoretically possible. Indeed, such a possibility has been postulated for flavin binding in other flavoproteins, *e.g.*, Old Yellow Enzyme (Theorell and Nygaard, 1954b) and cytochrome b_5 reductase (Strittmatter, 1961). Nevertheless, with *Azotobacter* apoprotein a definitive answer to this problem must await further investigation.

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