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Studies with Flavin Analogs Provide Evidence That a Protonated Reduced FMN Is the Substrate-induced Transient Intermediate in the Reaction of *Escherichia coli* Chorismate Synthase*

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Chorismate synthase catalyzes the 1,4-elimination of phosphate and the C-(6-pro-*R*) hydrogen from 5-enolpyruvylshikimate 3-phosphate (EPSP) to generate chorismate. Although this reaction does not involve an overall change in redox state, the enzyme requires reduced FMN. To investigate the role of the flavin in catalysis we have employed chemically modified flavins: 1- and 5-deaza-, 2- and 4-thio-, 6-hydroxy-, 8-nor-6-methyl-, 8-methyl-sulfonyl-, 8-chloro-, 8-fluoro-, 8-nor-methyl-, 8-S-methyl-, 8-methoxy, 8-mercapto- and 8-amino-FMN. Photoreduction of 4-thio-FMN in the presence of chorismate synthase at pH 7.5 produced a reduced flavin species with an absorbance maximum at λ = 410 nm indicative of monoanionic, reduced 4-thio-FMN. Binding of 8-mercapto- and 6-hydroxy-FMN to chorismate synthase in the presence of EPSP or (6*R*)-6-fluoro-EPSP resulted in an increase of the flavin analogs' pK_a values by 4 and 1 pH units, respectively. On the basis of these findings it is concluded that chorismate synthase preferentially binds neutral flavin species, including the protonated reduced form, rather than anionic flavin species in the presence of EPSP or the 6-fluoro-substrate analog. Further support for this conclusion was obtained using 5-deaza- and 4-thio-FMN. Addition of EPSP to enzyme-bound, reduced 5-deaza-FMN produced spectral changes consistent with protonation of the flavin. Photoreduction of 4-thio-FMN in the presence of enzyme and the (6*R*)-6-fluoro-EPSP generated a reduced flavin species with absorbance properties of a neutral, reduced 4-thio-flavin. These results and their implications for the nature and kinetic properties of an observed flavin intermediate are discussed in the context of a possible role of reduced flavin as an electron donor to bound EPSP.

Chorismate synthase (EC 4.6.1.4) catalyzes the 1,4-*anti*-elimination of phosphate from 5-enolpyruvylshikimate 3-phosphate (EPSP)[1] to yield chorismate (see Scheme 1).

This reaction does not involve an overall change in redox states. Therefore, an intriguing feature of this reaction is its requirement for reduced FMN, a cofactor widely utilized in enzyme-catalyzed redox reactions. A recent investigation of the rapid reaction kinetics of this enzyme revealed transient spectral changes indicative of a flavin intermediate (1, 2). However the spectral features of this intermediate are quite unusual and different from those found for other enzyme-bound flavin intermediates. The chemical nature of this intermediate is still uncertain.

In contrast to bifunctional chorismate synthases, *e.g. Neurospora crassa*, the enzyme from *Escherichia coli*, used in the present investigation, does not possess a flavin reductase activity (3), and hence reduction of FMN *in vivo* is possibly achieved by an accessory reductase at the expense of reduced NAD(P).

Chorismate synthase from *E. coli* bears some similarities with bacterial luciferase in that it has a higher affinity for the two-electron reduced form of FMN (4, 5). Moreover, reduction of the flavin in the presence of EPSP or (6*R*)-6-fluoro-EPSP and stoichiometric amounts of chorismate synthase leads to a stable blue (neutral) flavin semiquinone species. This radical species is similar to that obtained with flavodoxins (6) and bacterial luciferase (7). In addition, reduced FMN bound to chorismate synthase has a redox potential similar to flavins bound to flavodoxins (5).

Chemical modifications of the redox active isoalloxazine ring of FMN and FAD have been successfully used to probe the mechanism of action of several other flavoproteins and to characterize their flavin binding sites (8). 8-Mercaptoflavin was particularly useful in these investigations. We therefore decided to investigate and characterize the effects of flavin modifications on the spectral and catalytic properties of chorismate synthase in order to determine the role of reduced flavin in catalysis. In this context one major purpose of the present investigation was to elucidate the chemical structure of the observed intermediate. The chemical structures of the flavin analogs employed in the present investigation are summarized in Table I. Recently we reported that chorismate synthase binds reduced FMN in its monoanionic form, but in the presence of EPSP or (6*R*)-6-fluoro-EPSP the neutral form of the flavin appears to be preferentially stabilized by the enzyme (5). Therefore we concluded that chorismate synthase-bound, reduced, monoanionic FMN becomes protonated when the substrate binds to form a ternary complex. Here we present further evidence that reduced flavin is bound to chorismate

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This work is dedicated to Prof. Dr. Vincent Massey on the occasion of his 70th birthday.

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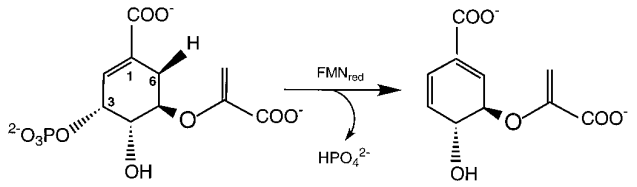
[1] The abbreviations used are: EPSP, 5-enolpyruvylshikimate 3-phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid.



SCHEME 1. **Reaction catalyzed by chorismate synthase.**

synthase in its monoanionic form and that protonation occurs at the N(1)–(C=O) locus of FMN upon binding of either EPSP or (6*R*)-6-fluoro-EPSP to the binary complex. In agreement with results from a kinetic and transient spectral analysis, reported from this laboratory (9), our results indicate that protonated, reduced FMN is the observed reaction intermediate.

MATERIALS AND METHODS

Chemicals—Glycerol was from BDH Laboratory Supplies, Poole, United Kingdom. MOPS, cellulose phosphate (medium mesh), DEAE-Sephacel, flavin mononucleotide (FMN, 95%), and riboflavin were from Sigma, Poole, U.K. (6*R*)-6-Fluoro-EPSP was prepared as recently described (5), and the potassium salts of EPSP and (6*R*)-6-fluoro-EPSP were prepared according to Knowles *et al.* (10).

Enzymes—Chorismate synthase was purified from an overproducing strain of *E. coli* (AB2849/pGM605) as described recently (11). From 20 g of cell paste we typically obtained 125 mg of enzyme with a purity of >95% as judged by SDS-polyacrylamide gel electrophoresis. The enzyme was stored in liquid nitrogen.

Flavin Analogs—The FMN derivatives used in this work were obtained by conversion of the corresponding riboflavin to the FAD level using the purified FAD-synthetase complex of *Brevibacterium ammoniagenes* followed by the treatment with phosphodiesterase from *Naja naja siamensis*. (12). They are listed in Table II, along with the references pertaining to their preparation and redox potentials. 8-Mercapto-FMN was synthesized from 8-chloro-FMN by addition of 10 mM sodium sulfide (13). Freshly prepared 8-mercapto-FMN was used immediately or stored in liquid nitrogen.

Activity Assays—The flavin analogs used in this study were tested for activity in the chorismate synthase assay described by Ramjee *et al.* (14) with the following modifications. A quartz cuvette fitted with a side arm was employed. The cuvette contained the flavin (5–10 μM), EPSP (90 μM), and potassium oxalate (1 mM) in 50 mM MOPS buffer, 10% glycerol, pH 7.5 (total volume 1 ml). The enzyme was placed in the side arm (5 μl of a 23 μM chorismate synthase solution yielding a final concentration of 0.11 μM). After sealing the cuvette, anaerobiosis was established by several cycles of evacuation and flushing with dinitrogen. Flavin reduction was achieved by 3 min of light irradiation. The cuvette was then thermostatted at 25 °C, and a base line at 275 nm was recorded. The enzyme was quickly mixed with the sample, and the absorbance changes at 275 nm ($\Delta\epsilon$ = 2630 M^{-1} cm^{-1}) were monitored with a Shimadzu spectrophotometer (model MPS-2000) equipped with a Shimadzu graphic printer (model PR-3).

Photoreduction—Samples were made anaerobic by several cycles of evacuation and flushing with dinitrogen or argon. Reduction was then achieved by irradiation of the solution (containing 1 mM potassium oxalate) with white light (150-watt halogen bulb) using an illumination system (model KL 1500, Schott, Mainz, Germany) equipped with two fiber optic light guides.

UV/Vis Absorbance Spectrophotometry—Absorbance spectra were recorded with an HP photodiode array instrument (model HP8452).

Rapid Reaction Spectrophotometry—Formation and decay of the flavin intermediate was observed using a Hi-Tech Scientific SF-51 stopped-flow spectrophotometer (Salisbury, U.K.) installed and operated in an anaerobic glove box as described earlier (9, 15). Spectra of the intermediate were recorded using a Hi-Tech Scientific MG-6000 rapid scanning photodiode array connected to the SF-51 stopped-flow handling unit. The integration time for collecting spectra between 350 and 600 nm was 10 ms. Kinetic data were fitted with the Hi-Tech Scientific IS-2 v2.3b5 software.

Anaerobic solutions of the substrate and a stoichiometric concentration of enzyme and FMN were reduced either by sodium dithionite or photoirradation in the presence of potassium oxalate. All rapid reaction experiments were carried out in 50 mM MOPS, 10% glycerol, pH 7.5, at 25 °C.

Redox Titrations—The redox potential of iso-FMN in the presence of chorismate synthase was determined by the method recently described by Massey (16). The cuvette contained 21.5 μM chorismate synthase, 20.6 μM iso-FMN, 25 μM 2-hydroxy-1,4-napthoquinone (E_0' = −139 mV), 2 μM methyl viologen (E_0' = −440 mV), and 500 μM xanthine in 800 μl of 50 mM MOPS buffer, 10% glycerol, pH 7, at 25 °C. After anaerobiosis was established a catalytic amount of xanthine oxidase was added to this mixture, and the ensuing reduction of FMN and the dye was followed spectrophotometrically with time. For each time point, the concentration of oxidized and reduced FMN as well as oxidized and reduced dye was calculated, and the log ox/red for FMN was plotted against the log ox/red for the dye. The plots generated in this manner had unity slope as expected for a two electron/two electron reduction of FMN and napthoquinone.

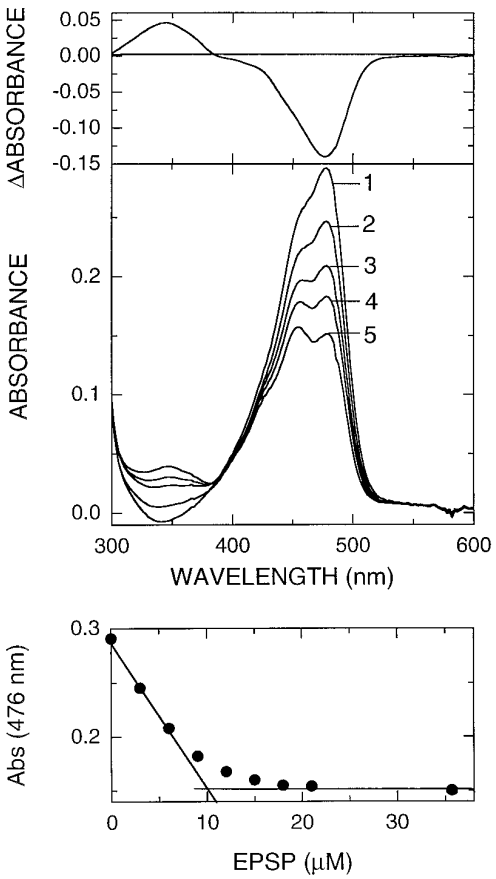


FIG. 1. **Binding of 8-S-methyl-FMN to chorismate synthase.** The spectral changes occurring upon addition of EPSP to 8-S-methyl-FMN (10.4 μM) and chorismate synthase (14.3 μM) are shown in the *center panel*. The spectra were recorded at 0, 3, 6, 9, and 35.7 μM EPSP (*traces 1–5*). The *top panel* represents the difference between spectrum 1 and 5. The absorbance changes at 476 nm at 0, 3, 6, 9, 12, 15, 18, 21, and 35.7 μM EPSP are shown in the *lower panel*. The titration was performed in 50 mM MOPS, 10% glycerol, pH 7.5 at 25 °C.

RESULTS

Binding and Activity of 8-Substituted Flavin Analogs

Addition of chorismate synthase to the oxidized form of the 8-substituted FMN derivatives listed in Table II did not cause spectral perturbation (experimental conditions as in Figs. 1 and 2 but without EPSP). The lack of spectral changes in the absence of EPSP reflects the weak binding of oxidized FMN to the enzyme as recently reported in detail (4, 5). In the presence of EPSP, however, spectral perturbations were observed for all flavin derivatives. The most pronounced changes were found for 8-S-methyl-, 8-chloro-, 8-methoxy-, and 8-amino-FMN. The spectral perturbations associated with binding of 8-S-methyl- and 8-Cl-FMN are depicted in Figs. 1 and 2, respectively. Only small spectral changes were observed for 8-H-, 8-F-, and 8-SO_2-CH_3-FMN (not shown). The spectral changes observed with

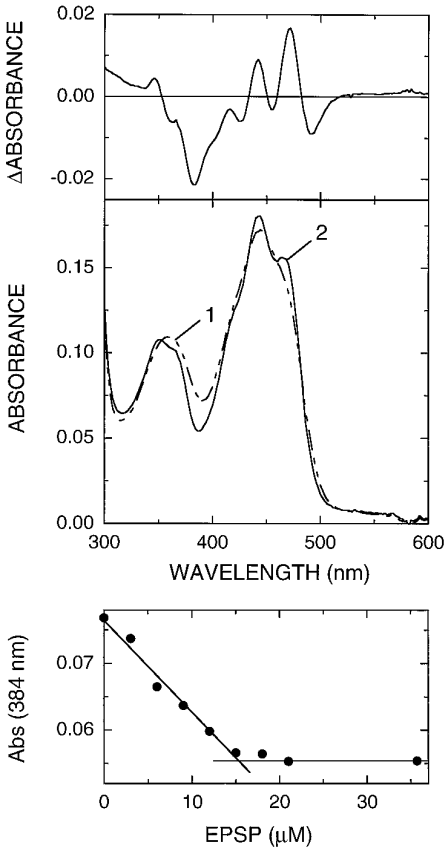


FIG. 2. **Binding of 8-Cl-FMN to chorismate synthase.** The *center panel* shows the spectral changes associated with the binding of 8-chloro-FMN (14.9 μM) to chorismate synthase (17.2 μM). The *solid line* represents the spectrum of FMN in the absence of and the *dashed line* in the presence of EPSP (35.7 μM). The *top panel* depicts the difference spectrum. The *lower panel* represents the absorbance at 384 nm at 0, 3, 6, 9, 12, 15, 18, 21, and 35.7 μM EPSP. All spectra were recorded in 50 mM MOPS, 10% glycerol, pH 7.5 at 25 °C.

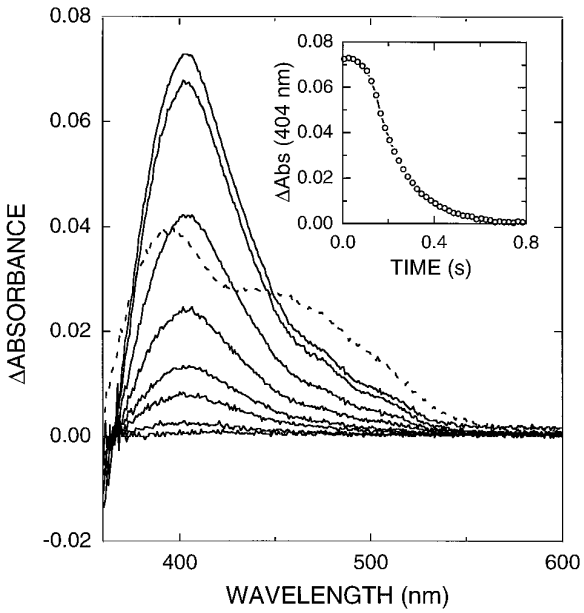


FIG. 3. **Single turnover experiment with 8-S-methyl-FMN.** An anaerobic solution of chorismate synthase and 8-S-methyl-FMN (both 40 μM) was mixed with EPSP (40 μM) in the stopped-flow instrument. Both solutions were reduced with sodium dithionite (1.6 mM). The spectra were recorded after 30 (*top spectrum*), 110, 190, 270, 350, 430, 590, and 790 ms (10 ms integration time). The transient spectrum with FMN is also shown for comparison (*dashed line*). The *inset* shows the absorbance changes at 404 nm as a function of time. The experiment was performed in 50 mM MOPS, 10% glycerol, pH 7.5 at 25 °C.

8-mercapto-FMN will be discussed separately in the next section.

Dissociation constants (K_D) and activities for the 8-substituted flavins used in this investigation are summarized in Table II. The K_D values found for the 8-substituted flavins vary between 8.5 and 0.5 μM for 8-amino- and 8-S-methyl-FMN, respectively, and are higher than recently found for FMN (5). Due to the observed 1:1:1 stoichiometry in the titration experiments, dissociation constants for EPSP binding to the FMN analog-enzyme complex must be in the same range as those for the FMN analogs.

With both 8-Cl- and 8-F-FMN bound to chorismate synthase (in the presence of EPSP), no spontaneous displacement of the halogen was observed suggesting that none of the four thiol groups in chorismate synthase are in the vicinity of the 8-position of the isoalloxazine ring system. The reaction of thiophenol with the 8-Cl-FMN was very slow compared with that of the free 8-Cl-FMN (15 M^{-1} min^{-1} when chorismate synthase-bound and 3200 M^{-1} min^{-1} for free 8-Cl-FMN (17)). The second order rate constant for the reaction of the bound 8-Cl-FMN with sulfide was found to be 1 M^{-1} min^{-1} as compared with 5.12 M^{-1} min^{-1} for free 8-Cl-FMN (17). The course of the latter reaction was unusual in that sulfide caused rapid bleaching of the absorbance at 450 nm with only a small increase of absorbance due to the formation of 8-mercapto-FMN. This indicates that nucleophilic attack on the 8-position leads to a metastable tetrahedral complex which then, in a second reaction phase, decays to yield the final product 8-mercapto-FMN. Similar heterogeneities of the reaction between 8-Cl-flavins and sodium sulfide were reported by Schopfer *et al.* (17). The second order rate constant of 1 M^{-1} min^{-1} reported above takes only the first rapid phase of the reaction into account.

The activities found in the presence of flavin-reduced 8-substituted flavins vary considerably, but there appears to be no obvious correlation between the activity and the redox potential of the flavin derivative (Table II). In addition, some flavin derivatives exhibit a strong effect on the K_m of EPSP. Recently, Ramjee *et al.* (14) reported a K_m for EPSP of 1.3 μM. As a consequence the rate of conversion of EPSP to chorismate is linear during most of the reaction using the assay described by Ramjee *et al.* (14) until most of the EPSP is consumed and the concentration approaches the K_m. This behavior was also found for 8-methoxy-, 8-S-CH_3-, and 8-H-FMN suggesting a similarly low K_m for EPSP. With all other 8-substituted flavins, however, the assays were nonlinear at the concentrations used indicating that the substitution in the 8-position caused an increase of the K_m. The activities given in Table II are therefore the initial rates observed at the EPSP concentration used in the assays (=90 μM).

Spectral Characterization of the Flavin Intermediate Using 8-S-Methyl-FMN

Ramjee *et al.* (1) reported a flavin intermediate in the chorismate synthase-catalyzed conversion of EPSP to chorismate. To further characterize the nature of this intermediate 8-S-methyl-FMN was chosen for two reasons. First, steady-state assays, as described in the previous section, revealed that the K_m for EPSP was not greatly affected by the substitution in the 8-position; and second, the spectral features of oxidized 8-S-CH_3-FMN are quite distinct from those of oxidized FMN, whereas the absorbance spectra of these reduced flavins are very similar. As shown in Fig. 3, the transient difference spectrum obtained with the 8-S-methyl-FMN has an absorbance maximum at 403 nm with a maximal absorbance change of

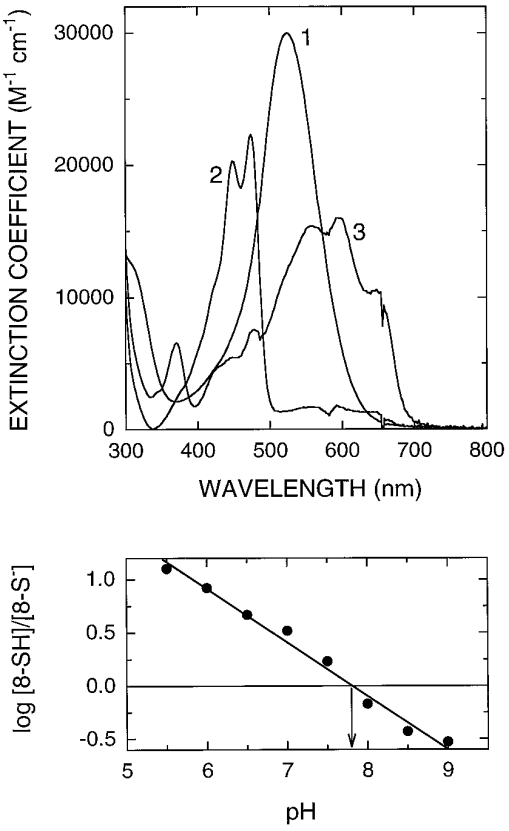
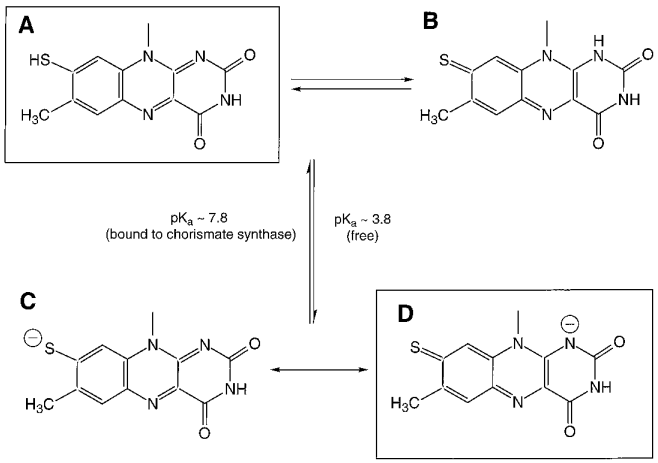


FIG. 4. **UV/Vis absorbance spectra of 8-mercapto-FMN bound to chorismate synthase.** The spectra are of free 8-mercapto-FMN (*trace 1,* in 50 mM MOPS buffer, pH 7.5, containing 10% glycerol) and the enzyme-bound 8-mercapto-FMN at pH 6 (*trace 2,* in 50 mM MOPS buffer, 10% glycerol) and pH 9 (*trace 3,* in 50 mM Tris buffer, 10% glycerol). All the spectra were recorded at 25 °C. Samples were prepared as follows: 11.5 μM chorismate synthase, 11.3 μM 8-mercapto-FMN, and 30 μM EPSP (in 1 ml) were incubated for 15 min at 25 °C before any unbound 8-mercapto-FMN was removed using microconcentrators (Centricon with a cut-off of 30 kDa). The concentrated protein fractions were then rediluted with buffer to a final volume of 0.8 ml. The points shown in the *bottom graph* were obtained at pH 5.5, 6.0, 6.5, 7.0, 7.5 (all in MOPS buffer), 8.0, 8.5, and 9.0 (all in Tris buffer).

A_{403} = 0.073. This is similar but not identical to the difference spectrum reported for FMN that has an absorbance maximum at 395 nm and a smaller maximal absorbance change of A_{395} = 0.04 (11, 18). With both flavins the formation of the intermediate was very rapid and occurred almost within the dead time of the stopped-flow instrument (~4 ms). The decay of the intermediate using 8-S-methyl-FMN was found to proceed with a first order rate constant of 6.9 s^{-1} (average of four independent experiments). This decay rate was fitted using a single exponential on the lower half of the decay curve. With FMN this rate was substantially faster with an observed rate constant of 52 s^{-1}. Similarly, V_{max} derived from steady-state assays was affected by a similar amount (see Table II).

Flavin Analogs with Ionizable Groups

8-Mercapto- and 6-Hydroxy-FMN—As reported recently for FMN (4, 5), addition of stoichiometric amounts of chorismate synthase to 8-mercapto-FMN does not perturb the UV/Vis absorbance spectrum (Fig. 4, *trace 1* shows the spectrum of free 8-mercapto-FMN). However, addition of EPSP leads to major changes in the UV/Vis absorbance spectrum of 8-mercapto-FMN that are strongly dependent on the pH (Fig. 4). At low pH the spectrum of the 8-mercapto-FMN exhibits two peaks at 448 and 474 nm (Fig. 4, *trace 2*), whereas at high pH a rather broad absorbance is observed with maxima at 556 and 594 nm (Fig. 4, *trace 3*). Comparison with UV/Vis absorbance spectra of other flavoproteins reconstituted with 8-mercaptoflavins clearly shows that the absorbance spectrum at low pH is similar to that reported for the neutral 8-mercapto-FMN species (see Scheme 2, *structure A*), as was also found for 8-mercaptoriboflavin bound to riboflavin binding protein (19). At high pH, however, the observed UV/Vis absorbance spectrum has the features of the paraquinoid form of the deprotonated 8-mercapto-FMN species (Scheme 2, *structure D*).



SCHEME 2. **Possible tautomeric and mesomeric structures of 8-mercaptoflavin (adopted from Ref. 19).** The *boxed structures A* and *D* are preferentially bound to chorismate synthase at low and high pH, respectively.

The extinction coefficients of the 8-mercapto-FMN bound to chorismate synthase at low (6.0) and high pH (9.0) were determined by denaturation of chorismate synthase-bound 8-mercapto-FMN with 6 M guanidinium hydrochloride. Using an extinction coefficient of 28,000 M^{-1} cm^{-1} at 530 nm for free 8-mercapto-FMN (19), extinction coefficients of 24,250 M^{-1} cm^{-1} at 476 nm and 15850 M^{-1} cm^{-1} at 594 nm were obtained for the neutral (at pH 6) and anionic protein-bound 8-mercapto-FMN (at pH 9) species, respectively. These extinction coefficients were used to calculate the concentration of enzyme-bound neutral and anionic 8-mercapto-FMN at various pH values. As shown in Fig. 4 (*bottom*), a pK_a value of 7.8 was found for the deprotonation of the chorismate synthase-bound 8-mercapto-FMN. This pK_a is 4 units higher than that found for free 8-mercapto-FMN (19) and represents a very large shift of the pK_a that is only exceeded by that observed for riboflavin binding protein (19).

Recently, we have reported that the binding of FMN to chorismate synthase is much tighter in the presence of EPSP (20 nM (5)). A similar but less pronounced effect has been found with 8-mercapto-FMN. The observed K_D values in the presence of substrate were 0.5 μM (pH 6.0) and 1.4 μM (pH 9.0), *i.e.* 25 to 75 times higher than the K_D determined for FMN (pH 7.5).

8-Mercapto-FMN in its oxidized form was inactive in the enzyme-catalyzed conversion of EPSP to chorismate. The reduced species, however, showed appreciable activity amounting to approximately 30% of the activity with FMN (see Table II).

Titration of oxidized 6-hydroxy-FMN in the presence of chorismate synthase with substrate gives rise to the spectral changes shown in Fig. 5 (*top graph*). At pH 7.5 the absorbance at 600 and 335 nm decreases, whereas the absorbance at 420 nm is essentially unchanged. These spectral changes indicate that 6-hydroxy-FMN is protonated upon binding of EPSP to chorismate synthase. As shown in the *inset* of Fig. 5, the pH dependence of the absorbance at 600 nm indicates a pK_a of 8 for 6-hydroxy-FMN bound to chorismate synthase. This is ~1 pH

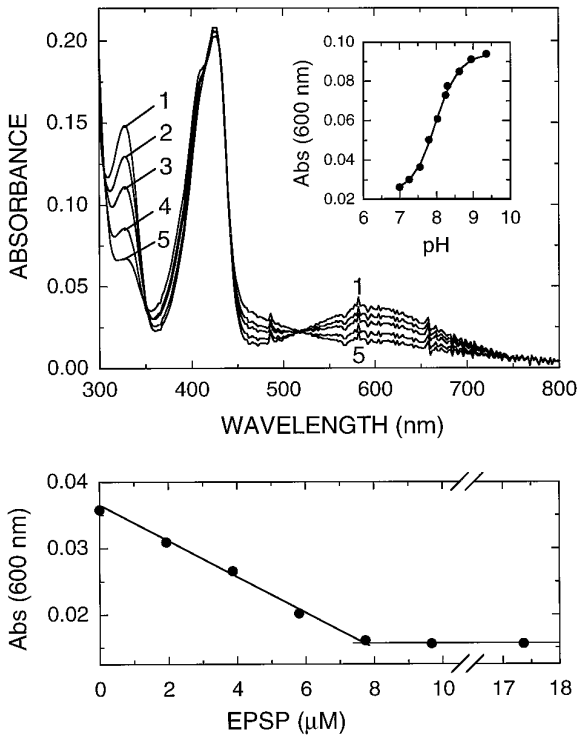


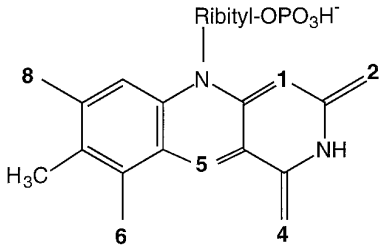
FIG. 5. **The binding of 6-OH-FMN to chorismate synthase.** The spectral changes upon addition of EPSP to a mixture of 10.4 μM chorismate synthase and 9.5 μM 6-OH-FMN are shown in the *upper graph* (50 mM MOPS, 10% glycerol, pH 7.5, 25 °C). The spectra shown were recorded at 0, 1.9, 3.9, 5.8, 9.7, and 17.4 μM EPSP. The *inset* shows the absorbance at 600 nm as a function of pH. The pH titration was carried out in 50 mM Tris, and the pH was adjusted by addition of solid Tris or MOPS. A fit to the observed data points is represented by the *solid line* yielding a pK_a of 8. The *lower panel* depicts the absorbance changes at 600 nm upon titration with EPSP.

unit higher than that found for free oxidized 6-hydroxy-FMN (20). The upward shift found for chorismate synthase-bound 6-hydroxy-FMN is not as pronounced as that reported for flavodoxin (pK_a = 9; (20)) and clearly not as dramatic as the pK_a shift for chorismate synthase-bound 8-mercapto-FMN (see above). In the presence of substrate, 6-hydroxy-FMN binds very tightly to chorismate synthase with an estimated K_D of 55 nM (see also titration in Fig. 5, *bottom graph*).

8-Nor-6,7-dimethyl-FMN (Iso-FMN)—Titration of iso-FMN in the presence of chorismate synthase with EPSP produced only small spectral perturbations of the iso-FMN UV/Vis absorbance spectrum. After filtration in a microconcentrator (Centricon 30) most of the iso-FMN was found in the filtrate, and a K_D of 5 μM was determined. This K_D is 250 times larger than the one recently reported for FMN (5) indicating that the methyl group in 6-position interferes with binding to the enzyme-substrate complex (see Table I).

In the presence of the enzyme, the redox potential of iso-FMN was determined to be E_0' = −137 mV, *i.e.* 63 mV more positive than that of free iso-FMN (E_0' = −200 mV (21)). This amounts to a 135-fold tighter binding of reduced iso-FMN compared with the oxidized form. This compares with a 1660-fold tighter binding of reduced FMN than oxidized FMN (5), *i.e.* the relative binding of the reduced *versus* the oxidized form is 12 times higher for FMN than with iso-FMN. However, the binding of the oxidized iso-FMN in the presence of substrate is affected to a much larger extent (250-fold) than is the reduced state, indicating that the methyl group in the 6-position interferes less with the binding of the reduced flavin. In view of these findings it was not surprising that reduced iso-FMN showed very little activity amounting to 0.15% of that of reduced FMN (see Table II). Photoreduction under anaerobic conditions of iso-FMN in the presence of chorismate synthase and a 2-fold excess of (6*R*)-6-fluoro-EPSP led to the formation of the neutral blue flavin semiquinone as was previously reported for FMN (5). However, based on the extinction coefficient reported for the iso-FMN semiquinone radical species bound to apoflavodoxin of ϵ = 2700 M^{-1} cm^{-1} (22), the yield of radical was only 55%, whereas with FMN approximately 100% radical was formed during the course of photoreduction (5). Moreover, no radical was observed when a 3-fold excess of EPSP was added to an anaerobic solution of photoreduced iso-FMN and chorismate synthase. Under similar experimental conditions full formation of the neutral flavin semiquinone was observed (5).

TABLE I



	POSITION					
	1	2	4	5	6	8
FMN	N	O	O	N	H	CH_3
1-Deaza-FMN	C	O	O	N	H	CH_3
2-Thio-FMN	N	S	O	N	H	CH_3
4-Thio-FMN	N	O	S	N	H	CH_3
5-Deaza-FMN	N	O	O	C	H	CH_3
Iso-FMN	N	O	O	N	CH_3	H
6-Hydroxy-FMN	N	O	O	N	OH	CH_3
6-Amino-FMN	N	O	O	N	NH_2	CH_3
8-H-FMN	N	O	O	N	H	H
8-F-FMN	N	O	O	N	H	F
8-Cl-FMN	N	O	O	N	H	Cl
8-Mercapto-FMN	N	O	O	N	H	SH
8-S-CH_3-FMN	N	O	O	N	H	SCH_3
8-SO_2CH_3-FMN	N	O	O	N	H	SO_2CH_3
8-Methoxy-FMN	N	O	O	N	H	OCH_3
8-Amino-FMN	N	O	O	N	H	NH_2

1- and 5-Deaza-FMN—Chorismate synthase from *N. crassa* and *E. coli* reconstituted with reduced 5-deaza-FMN were reported to be inactive (23, 24). The absence of activity is not due to a lack of binding of the 5-deaza-FMN to the enzyme since, as is shown in Fig. 6, 5-deaza-FMN binds tightly to *E. coli* chorismate synthase in the presence of EPSP, as indicated by the pronounced spectral changes that lead to a highly resolved flavin spectrum. Similar spectral changes were recently reported for the binding of FMN (5). Reduction of 5-deaza-FMN in the presence of chorismate synthase caused complete bleaching of the absorbance band peaking at 400 nm. The reduced 5-deaza-FMN exhibits an absorbance peak at 318 nm (Fig. 7) that is characteristic of the 1,5-dihydro-form of 5-deaza-FMN (25). Addition of EPSP to the enzyme-bound reduced form resulted in a shift to longer wavelength as indicated in Fig. 7, *trace 3*. The observed spectral changes upon binding of EPSP are in fact very similar to those seen on protonation of the anionic 1,5-dihydro-5-deaza-FMN reported by Spencer *et al.* (25). These authors report a shift of the absorbance maximum from 319 to 323 nm with a slightly higher extinction coefficient for the protonated species. Since the pK_a = 7.2 of free reduced 5-deaza-FMN (25) is close to that found for free reduced FMN (pK_a = 6.7, (26)), this result provides further evidence that protonation of the enzyme-bound reduced FMN occurs upon binding of the substrate, EPSP.

Oxidized 1-deaza-FMN was the only FMN derivative found

TABLE II

FMN derivative	K_D[a]	Activity[b]		Redox potential[c]	Synthesis
		nmol min^{-1}	%		
	μM			E_m *in mV*	
8-SO_2-CH_3	8.5	2.8	4.5	−50 (13)	13
4-Thio	0.45	[e]	~2[e]	−55 (27)	27
2-Thio	2.9	27.9	44	−120 (30)	31
8-Cl	0.7	ND[f]	ND[f]	−150 (30)	32
8-F	20	ND[f]	ND[f]	−167 (33)	34
8-H	4	32.9	52	−180 (35)	36
Iso	5	<0.063[g]	<0.1[g]	−200 (21)	37
8-SCH_3	0.5	8.2	13	−204 (13)	13
FMN	0.02	63.3	100	−205 (38)	
6-Hydroxy	0.05	<0.063[g]	<0.1[g]	−255 (16)	39
8-Methoxy	0.63	48.7	77	−260 (40)	40
1-Deaza	<0.25	9.5	15	−280 (41)	42
8-Mercapto	0.5–1.4[d]	19	30	−290 (13)	19
6-Amino	0.3	<0.063[g]	<0.1[g]	−293 (43)	43
8-Amino	2	10.1	16	−310 (30)	44
5-Deaza	0.2	[h]	<1[h]	−311 (41)	25
Riboflavin	560	3.8	6	−199 (38)	

[a] K_D value for oxidized forms were measured in the presence of a 2-fold excess of EPSP.
[b] Enzyme activity was determined using the assay described under "Materials and Methods." Reduction of 6-hydroxy- and 6-amino-FMN was achieved using dithionite instead of light/oxalate; activity with FMN was set to 100%.
[c] Midpoint potentials for free flavins under standard conditions are listed.
[d] K_D is pH-dependent: 0.5 μM at pH 6 and 1.4 μM at pH 9.
[e] Assays with 4-thio-FMN were extremely curved and did not proceed to completion.
[f] ND, not determined.
[g] Detection limit: ~0.1% (=0.063 nmol/min) of FMN activity.
[h] Ref. 23.

to bind more tightly to chorismate synthase than oxidized FMN in the absence of EPSP (K_D = 6.8 μM). Addition of EPSP further decreased the K_D to below 0.24 μM. Binding of 1-deaza-FMN to chorismate synthase is accompanied by only small spectral changes (data not shown). Addition of EPSP does not cause further spectral changes. Unlike with 5-deaza-FMN, a significant activity was observed for 1-deaza-FMN that was ~15% of that found for FMN. However, the activity was strongly dependent on the concentration of EPSP as was already described for other flavin derivatives (see above).

4-Thio-FMN—4-Thio-flavins are probes for the state of protonation of reduced flavins by virtue of the large separation of the long wavelength absorbance maxima for the protonated (λ_{max} = 485 nm) and deprotonated (λ_{max} = 425 nm) reduced species (27). In addition, reduced 4-thio-flavin also exhibits a much lower pK_a of 4.5 as compared with 6.7 for FMN for the deprotonation of the N(1)-proton (27). As shown in Fig. 8 (*spectrum 2*), when 4-thio-FMN was reduced in the presence of chorismate synthase the resulting spectrum of 4-thio-FMN has an absorbance maximum at 410 nm clearly indicating that chorismate synthase stabilizes the deprotonated form of 4-thio-FMN at pH 5.8. This hypsochromic shift of 15 nm as compared with the free reduced 4-thio-FMN also demonstrates that chorismate synthase significantly affects the spectral properties of bound reduced flavins. Oxidized 4-thio-FMN binds with a K_D of 0.45 μM to chorismate synthase in the presence of EPSP. The spectral changes occurring upon binding of oxidized 4-thio-FMN to the enzyme are shown in Fig. 8, *spectrum 3.* Binding is accompanied by a slight shift of the absorbance maximum at 498 nm and a decrease of the extinction coefficient of this longer wavelength absorbance peak. Reduction of 4-thio-FMN in the presence of chorismate synthase and the substrate analog (6*R*)-6-fluoro-EPSP leads to a reduced 4-thio-flavin with an absorbance maximum at 465 nm (Fig. 9). This result strongly suggests that in the presence of this substrate analog the

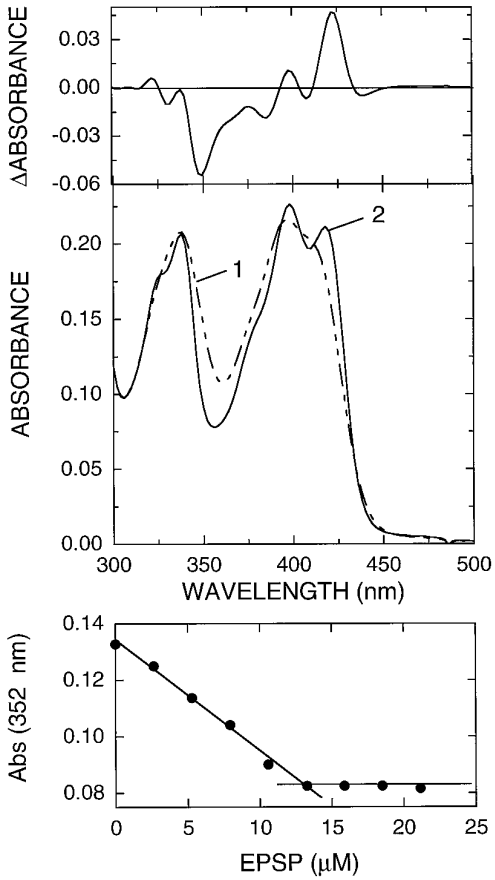


FIG. 6. **Binding of 5-deaza-FMN to chorismate synthase in the presence of EPSP.** Chorismate synthase (15.5 μM) and 5-deaza-FMN (16 μM) in 50 mM MOPS, 10% glycerol, pH 7.5, were titrated with EPSP at 25 °C. The UV/Vis absorbance spectra before (*solid line*) and after the addition of EPSP (15.9 μM, *dashed line*) are shown in the *center panel*. The *top panel* depicts the UV/Vis difference spectrum of the final spectrum and the starting spectrum. The *lower panel* shows the the absorbance decrease at 352 nm at 0, 2.6, 5.3, 7.9, 10.6, 13.3, 15.9 18.5, and 21.2 μM EPSP concentration

protonated form of reduced 4-thio-FMN is bound to chorismate synthase. It is also interesting to note that in the presence of the fluoro substrate analog only very little radical is formed during the course of the reduction depicted in Fig. 9. This contrasts with earlier observations reported by Macheroux *et al.* (5) that photoreduction of FMN in the presence of (6*R*)-6-fluoro-EPSP leads to complete formation of the blue neutral radical species with further reduction to the fully reduced species being extremely slow. Similarly, when EPSP was added anaerobically to chorismate synthase-bound, reduced 4-thio-FMN the blue neutral semiquinone was not observed even after prolonged incubation. Under identical conditions except with FMN, formation of the neutral semiquinone was observed (5, 18). With flavodoxin, on the other hand, formation of the 4-thio-FMN semiquinone was observed (27) indicating that the sulfur in the 4-position of the isoalloxazine ring system interferes with the stabilization of the neutral radical in chorismate synthase. Bulkier substituents in the 4-position, such as in 4-NOH-FMN, also led to the abolition of semiquinone stabilization in flavodoxin (27).

DISCUSSION

The UV-Vis absorbance spectrum of reduced FMN bound to chorismate synthase and the pH dependence of its redox potential have prompted us recently to suggest that reduced FMN is bound in its deprotonated, monoanionic form in the absence of substrate (5). However, the assignment of a state of proto-

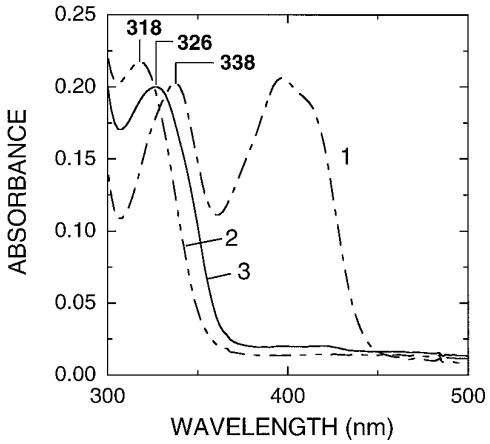


FIG. 7. **Reduction of 5-deaza-FMN in the presence of chorismate synthase and the effect of the subsequent addition of EPSP.** 5-Deaza-FMN (15.7 μM) and chorismate synthase (17 μM) in 50 mM MOPS, 10% glycerol, pH 7.5, was made anaerobic in a cuvette by repeated cycles of evacuation and flushing with nitrogen. The UV/Vis absorbance spectrum of the oxidized sample was then recorded (*trace 1*). A small amount of solid sodium borohydride was added to the solution to fully reduce the 5-deaza-FMN (*trace 2*). 10 μl of a 5. 3 mM EPSP solution was now added from the side arm of the cuvette (final concentration: 65 μM, *trace 3*).

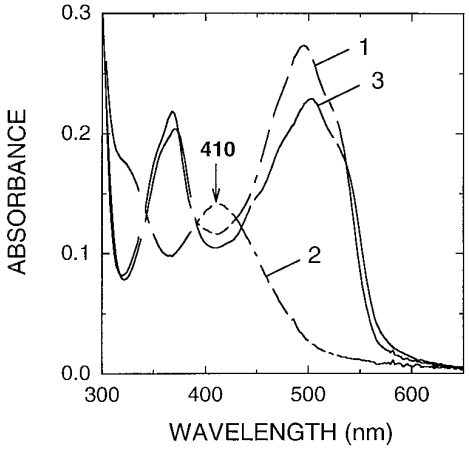


FIG. 8. **Binding of 4-thio-FMN to chorismate synthase.** A mixture of 17 μM 4-thio-FMN and 17.9 μM chorismate synthase in 50 mM MOPS, 10% glycerol at pH 5.8 was photoreduced under anaerobic conditions. The spectra of the oxidized and reduced 4-thio-FMN are represented by *traces 1* and *2*, respectively. *Trace 3* shows the spectrum of 4-thio-FMN bound to chorismate synthase in the presence of 16 μM EPSP.

nation to the reduced FMN is difficult since the anionic and neutral forms are distinguished by only subtle differences in their absorbance spectra. The analogous spectral differences for free 4-thio-FMN are much larger (λ_{max} = 425 and 485 nm for the anionic and neutral reduced forms, respectively) and therefore easier to interpret. In the absence of substrate, the spectrum of reduced 4-thio-FMN bound to chorismate synthase exhibits an absorbance maximum at 410 nm clearly indicating preferential binding of the monoanionic species (Fig. 8). It should also be noted that binding to chorismate synthase is accompanied by a 15-nm hypsochromic shift of the absorbance maximum. This shows that the protein environment substantially affects the spectral properties of reduced flavin species. Corroborating evidence for binding of the deprotonated reduced FMN was obtained from experiments with chorismate synthase-bound, reduced 5-deaza-FMN (Fig. 7) which exhibits an absorbance maximum at 318 nm very close to that reported for

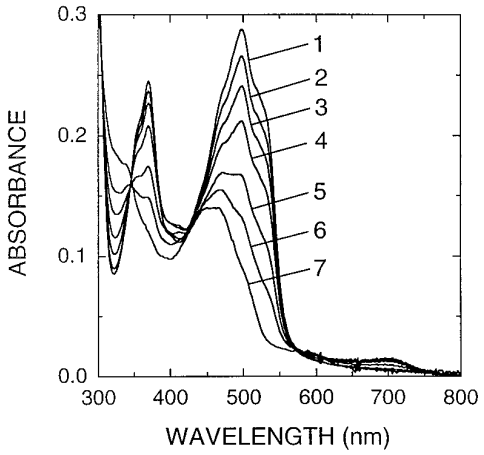


FIG. 9. **Photoreduction of 4-thio-FMN in the presence of (6*R*)-6-fluoro-EPSP.** 4-Thio-FMN (19.5 μM), chorismate synthase (23 μM), and (6*R*)-6-fluoro-EPSP (37.6 μM) were photoreduced under anaerobic conditions (50 mM MOPS, 10% glycerol, pH 7.5, and at 25 °C). *Spectrum 1* was recorded before photoreduction. *Spectra 2–7* were recorded after 10, 25, 55, 115, 235, and 535 s of light irradiation.

deprotonated free 5-deaza-FMN (λ_{max} = 319 nm (25)).

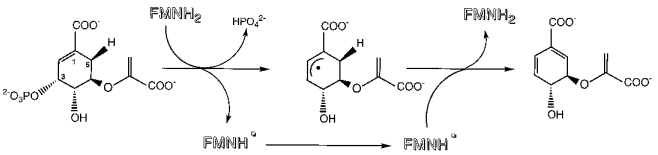
Recently, we have demonstrated that oxidized FMN binds much more tightly to chorismate synthase upon addition of EPSP or (6*R*)-6-fluoro EPSP. The binding of oxidized FMN was monitored using the spectral perturbations of the flavin absorbance (5). We have now further exploited these spectral changes in two ways. First, oxidized flavin derivatives with an ionizable group attached to the isoalloxazine ring were used as probes for the polarity of the flavin binding pocket. Second, reduced flavin derivatives were used to monitor spectral changes occurring upon binding of a substrate or substrate analog. The first group of flavin derivatives investigated carried an ionizable group at the 8- or 6-position (8-mercapto- and 6-hydroxy-flavin). Ghisla and Massey (8) have pointed out that these flavins mimic the deprotonation of the N(1)-proton of reduced FMN. As a consequence the shift in the pK_a of the ionizable group observed when the flavin derivative is bound to a flavoprotein reflects the shift of the pK_a of the N(1)-proton of reduced FMN. The large shift in the pK_a found for chorismate synthase-bound 8-mercapto-FMN in the presence of EPSP therefore suggests that the pK_a of the N(1)-proton of reduced FMN could be shifted upward, possibly by a similar order of magnitude, *i.e.* to a pK_a of ~11. The pK_a shift found for 6-hydroxy-FMN is not as pronounced as that for 8-mercapto-FMN and may reflect a greater hydrophobicity in the vicinity of the 8-position as compared with the 6-position. This is also consistent with the observation that chorismate synthase preferentially binds the neutral flavin semiquinone with a pK_a shifted upwards from 8.3 to at least 9.5 in the presence of EPSP (5). Direct evidence for protonation of chorismate synthase-bound, reduced flavins was obtained using 4-thio- and 5-deaza-FMN. As mentioned above, the difference in absorbance maxima of the protonated and deprotonated forms of reduced 4-thio-FMN allows conclusive assignment of the state of protonation. In the presence of (6*R*)-6-fluoro-EPSP (and pH 7.5) the absorbance maximum of the reduced 4-thio-FMN was at 465 nm, clearly shifted by 55 nm toward longer wavelengths as compared with chorismate synthase-bound 4-thio-FMN in the absence of the substrate analog. This bathochromic shift of the absorbance maximum is very similar to the 60-nm shift observed upon protonation of free reduced 4-thio-FMN (27). Hence, the pK_a of free reduced 4-thio-FMN (pK_a = 4.5 (27)) is increased by at least 3 pH units upon binding to chorismate synthase in the presence of the substrate analog. Surprisingly, the photoreduc-

tion shown in Fig. 9 also reveals that only small traces of neutral 4-thio-FMN radical were observed, in marked contrast to earlier experiments with FMN (5). In the case of flavodoxin, the semiquinone is observed with both FMN and 4-thio-FMN (27). However, bulkier substituents in the 4-position, such as the 4-aminomethyl group, led to the abolition of the stabilization of the flavin semiquinone. This was rationalized by the disruption of crucial hydrogen bonding interactions between the apoprotein and the N(5)-hydrogen of the isoalloxazine ring (27). Obviously, replacement of the oxygen by sulfur already compromises this interaction that is essential for stabilization of the neutral flavin semiquinone in the ternary complex of chorismate synthase, 4-thio-FMN, and the 6-fluoro-EPSP analog.

Additional evidence for the protonation of chorismate synthase-bound, reduced FMN comes from experiments with 5-deaza-FMN. This flavin was found to be inactive in the chorismate synthase-catalyzed conversion of EPSP to chorismate (23). Therefore, EPSP can be used instead of the 6-fluoro analog to demonstrate protonation of the reduced 5-deaza-FMN (Fig. 7). Spencer *et al.* (25) have observed a shift of the absorbance maximum from 319 to 323 nm upon protonation of free reduced 5-deaza-FMN. A similar bathochromic shift from 318 to 326 nm was observed upon addition of EPSP to chorismate synthase-bound, reduced 5-deaza-FMN. Since the pK_a = 7.2 of reduced 5-deaza-FMN (25) is very close to that of the reduced FMN (pK_a = 6.7 (26)), it can be regarded as a good model for FMN in respect to N(1)-protonation. This result again strongly supports our conclusion that substrate binding leads to protonation of the chorismate synthase-bound, reduced flavin.

It is generally accepted that the redox potential of a protein-bound flavin is linked to the pK_a for N(1) in the reduced form (8). A relatively positive redox potential is associated with a lower pK_a and vice versa. The former group of flavoproteins tends to stabilize the red anionic flavin radical (*e.g.* oxidases), whereas the latter group stabilizes the blue neutral semiquinone (*e.g.* flavodoxins). As suggested by Ghisla and Massey (8) this relationship between pK_a and the redox potential can be extended to flavin derivatives bearing an ionizable group, such as 8-mercapto- and 6-hydroxy-FMN. Accordingly, the pK_a shifts observed for 8-mercapto- and 6-hydroxy-FMN suggest that binding to chorismate synthase lowers the redox potential of the flavin comparable with that in flavodoxins. The extent of the shift is highly dependent on the position of the ionizable group indicating substantial differences between the flavin binding pocket in flavodoxin and chorismate synthase. In summary, the observed protonation of chorismate synthase-bound, reduced flavin upon substrate binding is a result of a substantial increase in the pK_a of the N(1) position, and this in turn is indicative of a much more negative redox potential. The physical origin of this decrease in redox potential is the change in the flavin environment, *i.e.* binding of substrate places the flavin in a less polar medium, and the associated desolvation leads to a destabilized reduced flavin and hence a more negative redox potential.

Substitution of the hydrogen by a methyl group in the 6-position of the flavin greatly affects its ability to bind to chorismate synthase. Weaker binding was observed for reduced iso-FMN both alone and in the ternary complex comprising enzyme, oxidized iso-FMN, and substrate. In addition, enzymatic activity was decreased dramatically. These results demonstrate that the bulky methyl group in 6-position of the flavin interferes with the formation of a catalytically competent ternary complex. This finding is in contrast to results obtained with a flavodoxin from *Megasphaera elsdenii* (22) and L-lactate monooxygenase from *Mycobacterium smegmatis* (28). In both



SCHEME 3.

cases, it was found that iso-FMN binds tightly to the apoproteins and that their catalytic properties were similar to those observed with FMN. Obviously both flavoproteins are able to accommodate the bulky methyl group, although in the case of L-lactate monooxygenase, a direct involvement of the adjacent N(5) in catalysis has been indicated. In contrast to iso-FMN, 6-amino- and 6-hydroxy-FMN bind more tightly to chorismate synthase in the presence of substrate (Table II). This indicates that the weak binding of iso-FMN is not solely a function of size but is also affected by the polarity of the substituent in the 6-position. The lack of the 8-methyl group may also contribute to the weaker binding of iso-FMN since deletion of the methyl group in 8-H-FMN substantially increased the K_D (Table II). All three 6-substituted FMN derivatives were incompetent in catalysis indicating that this region of the flavin cofactor plays a crucial role in catalysis. The low activities observed with 5-deaza-FMN (23, 24) and 4-thio-FMN (Table II) are also consistent with this conclusion.

All of the flavin derivatives studied showed weak binding to chorismate synthase in the absence of EPSP with the notable exception of 1-deaza-FMN (Table II). Oxidized 1-deaza-FMN binds ~5 times tighter to chorismate synthase than FMN (5). This contrasts to a ~100-fold weaker binding of oxidized 1-deaza-FMN in flavodoxins (29), again indicating differences in the flavin binding pocket of flavodoxins and chorismate synthase.

When 8-S-methyl-FMN was used as a cofactor, the formation of the transient spectral intermediate was, like with FMN, very fast and almost complete within the dead time of the stopped-flow instrument (4 ms). The decay of the intermediate, however, was 8 times slower than with FMN and correlates with the smaller V_{max}. The spectral features of the intermediate differ from those reported for FMN. The absorbance maximum is shifted from 395 to 404 nm with only a minor shoulder at longer wavelengths as compared with a dominant shoulder in case of FMN. In addition, the Δextinction coefficient found for the intermediate with 8-S-methyl FMN is slightly higher ($\Delta\epsilon_{max,\,404}$ = 4250 M^{-1} cm^{-1}). The effect of the chemical structure of the flavin on these transient spectral changes provides further support for the conclusion that the observed changes are associated with the flavin. In the kinetic experiments that led to the discovery of this intermediate, a stoichiometric concentration of reduced FMN and chorismate synthase, *i.e.* all FMN is bound to the enzyme, were rapidly mixed with EPSP. According to the conclusions of this paper, binding of the substrate to the chorismate synthase-bound, reduced FMN leads to protonation of the flavin. The spectral changes associated with the protonation of a monoanionic reduced flavin are consistent with the observed spectral properties of the intermediate. Three observations of the kinetic studies of the intermediate support this conclusion. 1) Formation of the intermediate is very rapid as can be expected for protonation (1, 9). 2) The occurrence of the intermediate precedes all chemical steps associated with the conversion of EPSP to chorismate, *i.e.* C–O and C–H bond cleavage occurs after formation of the intermediate. According to current kinetic models, close to 100% of the enzyme-bound flavin accumulates in the intermediate form during a single turnover reaction (9). 3) Dissociation of chorismate (probably one of the rate-limiting steps in the overall

reaction) appears to be associated with relaxation of the chorismate synthase-reduced FMN complex to the resting state, *i.e*. to the deprotonated reduced flavin species.

In summary, binding of EPSP to chorismate synthase-bound, reduced FMN causes a dramatic change in the binding of FMN from a polar to an apolar environment which brings about a more negative redox potential and stabilization of the flavin semiquinone species (5). Therefore, the properties of the bound reduced flavin are consistent with mechanistic proposals where the flavin plays a role as a one-electron donor (18). In this radical mechanism, C–O bond cleavage is associated with the formation of an allylic substrate-derived radical intermediate and a flavin radical. Decomposition of the allylic radical then provides an electron to regenerate reduced flavin and yields the product of the catalytic reaction (Scheme 3). This mechanism has recently been favored by kinetic studies (9). Hence, the substrate itself plays a crucial role in modulating the properties of reduced FMN, bound to chorismate synthase. Moreover, we propose that the properties of the reduced FMN are appropriate for the conversion of EPSP to chorismate only after the substrate is bound, in a process that could be termed *"substrate-induced cofactor activation."*

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