Binding of the Oxidized, Reduced, and Radical Flavin Species to Chorismate Synthase. An Investigation by Spectrophotometry, Fluorimetry, and Electron Paramagnetic Resonance and Electron Nuclear Double Resonance Spectroscopy†

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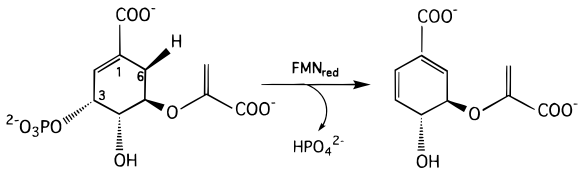
ABSTRACT: Chorismate synthase (EC 4.6.1.4) binds oxidized riboflavin-5′-phosphate mononucleotide (FMN) with a K_D of 30 μM at 25 °C, but in the presence of 5-enolpyruvylshikimate-3-phosphate (EPSP), the K_D decreases to *ca.* 20 nM. Similar effects occur with the substrate analogue (6*R*)-6-fluoro-EPSP (K_D = 36 nM) and chorismate (K_D = 540 nM). Fluorescence of oxidized FMN is slightly quenched in the presence of chorismate synthase. Addition of EPSP or the (6*R*)-6-fluoro analogue causes a shift of the fluorescence from 520 to 495 nm. Chorismate causes no shift in, but a quenching of, the fluorescence emission maximum. In the presence of EPSP, (6*R*)-6-fluoro-EPSP, or chorismate, the neutral flavinsemiquinone is generated. The electron paramagnetic resonance (EPR) line width of the flavin radical is indicative of a neutral flavinsemiquinone. Frozen solution electron nuclear double resonance (ENDOR) of the radical with (6*R*)-6-fluoro-EPSP shows a number of proton ENDOR line pairs. The largest splitting is assigned to a hyperfine coupling to the methyl group β-protons at position 8 of the isoalloxazine ring. The hyperfine-coupling (hfc) components have values of $A^{\perp}$ = 8.07 MHz and $A^{\parallel}$ = 9.60 MHz, giving A^{iso} of 8.58 MHz, consistent with a neutral semiquinone form. The isotropic hfc coupling of the 8-methyl protons with (6*R*)-6-fluoro-EPSP decreases by about 0.5 MHz when chorismate is bound, indicating that the spin density distribution within the isoalloxazine ring system depends critically on the nature of the ligand. The redox potential of FMN in the presence of chorismate synthase was 95 mV more positive than that of free FMN (at pH 7.0), equivalent to a 1660-fold tighter binding of reduced FMN. The pH dependence of the redox potential of chorismate synthase-bound FMN exhibits a slope of −30 mV per pH unit between pH 6 and 9, indicating that the two-electron reduction of the flavin is associated with the uptake of one proton; this, and the UV−visible spectrum, is consistent with the reduced flavin being bound to chorismate synthase in its monoanionic form.

Chorismate synthase (EC 4.6.1.4) catalyzes the conversion of 5-enolpyruvylshikimate-3-phosphate (EPSP)[1] to chorismate:

Scheme 1



It is the seventh and last enzyme of the shikimate pathway. Chorismic acid constitutes a major building block for the biosynthesis of an array of aromatic compounds, such as the aromatic amino acids. Since the enzymes of the shikimate pathway are only present in bacteria, fungi, and plants, but not in animals, they appear to be attractive targets for novel antibiotics and herbicides. Although this reaction does not involve a change in redox states, the enzyme requires reduced flavin for activity (Welch et al., 1974). A flavin cofactor is found, with only a few exceptions, in enzymes carrying out redox reactions. Therefore, the exact role of reduced flavin in the catalytic elimination of phosphate remains intriguing. Another surprising feature of the reaction is that the 1,4-elimination reaction proceeds with *anti* stereochemistry, although frontier orbital calculations (Fukui, 1965) and model studies (Hill & Bock, 1978) clearly favor *syn* stereochemistry for a concerted elimination reaction. Several mechanistic proposals have been put forward that attempt to reconcile the unexpected stereochemical course of the enzyme-catalyzed reaction with the predicted stereochemistry (Walsh et al., 1990). However, the role of the flavin in catalysis has not yet been addressed adequately, despite supporting evidence that reduced FMN may play an essential part in catalysis (Ramjee et al., 1991, 1992). Recently, Ramjee et

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[1] Abbreviations: FMN, riboflavin-5′-phosphate mononucleotide; EPSP, 5-enolpyruvylshikimate-3-phosphate; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; rf, radio frequency; hf(c), hyperfine (coupling).

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al. (1991) observed transient spectral changes during the catalytic conversion of EPSP to chorismate which were attributed to a flavin intermediate. In addition, the same authors discovered that (6*R*)-6-fluoro-EPSP, which does not undergo phosphate elimination by the enzyme to yield 6-fluorochorismate, induces the formation of the neutral (blue) flavinsemiquinone (Ramjee et al., 1992). Although the flavin radical was not characterized in detail, these authors suggest that a flavin radical may indeed be involved transiently in the catalytic process. However, chorismate synthase was found to bind oxidized flavin only very weakly (Ramjee et al., 1993) which results in its isolation as the flavin free apo-enzyme (White et al., 1988). It is therefore pertinent to the understanding of the role of the flavin in catalysis to investigate the factors involved in binding of the flavin to chorismate synthase. Here we report that two factors are mainly responsible for tighter binding of flavin, namely two-electron reduction of the flavin or formation of a ternary complex with either substrate or product. In the ternary complex, however, reduction of the flavin to the fully reduced form is hampered and stabilization of a neutral flavinsemiquinone is observed. The neutral flavinsemiquinone has been characterized in detail using UV−vis, EPR, and ENDOR spectroscopies.

ENDOR, with its ability to resolve small electron−nuclear hyperfine interactions which are often hidden within the EPR signal envelope, has been extensively used for the characterization of the semiquinoid radical of flavin model compounds (Eriksson et al., 1970; Müller et al., 1970; Kurreck et al., 1984; Möbius & Lubitz, 1987) and several flavoproteins in the neutral and anionic radical forms. These systems have been studied in liquid solutions as well as in disordered solids. The FMN radical, which has only a small *g* anisotropy for which almost no orientation selection is possible, generally yields "powder-type" hf patterns in the ENDOR as a result of the random orientation of the flavinsemiquinone in a polycrystalline sample. On the other hand, in liquid solution, where small molecules are allowed to tumble rapidly, anisotropic contributions are motionally averaged and only the isotropic contribution to the hyperfine interaction is observed. However, this is not the case when FMN is bound to an enzyme of high molecular weight, which prevents the aggregate from rapid tumbling so that the Redfield condition for fast molecular motion (Redfield, 1965) is no longer fulfilled. Thus, the FMN radical in frozen solution and even in liquid solution still often gives powder-type ENDOR spectra. Partial orientation selection has been observed for systems including the benzoquinone anion radical in disordered matrices (O'Malley & Babcock, 1986) and for the tyrosyl radical in ribonucleotide reductase (Bender et al., 1989). We have now shown that it can also be seen for the FMN radical bound to chorismate synthase. The magnitude of the hfc with the protons of the FMN 8-methyl group is a good indicator of the protonation state of the radical (Kurreck et al., 1984) and hence can be readily used to identify the anionic, neutral, and cationic forms of the flavinsemiquinone. This has been used in the present study to obtain further support for the assignment of the radical stabilized by chorismate synthase to a neutral flavinsemiquinone species for which interactions with different types of protons have been identified, namely, α-protons, *β*-protons, and matrix protons. These hfcs can serve as sensitive indicators of often subtle changes in the radical environment caused by, for example, the presence of either bound substrate or product affecting the unpaired electron spin density distribution of the flavinsemiquinone.

MATERIALS AND METHODS

Chemicals. Glycerol and 5,5′-indigodisulfonic acid (disodium salt) were purchased from BDH Laboratory Supplies, Poole, U.K. 3-(*N*-Morpholino)propanesulfonic acid (MOPS), cellulose phosphate (medium mesh), DEAE-Sephacel, methyl and benzyl viologen, xanthine, and flavin mononucleotide (FMN, 95%) were purchased from Sigma Chemical Co., Poole, U.K.

Synthesis of (6R)-6-Fluoro-EPSP. The starting material, (6*R*)-6-fluoroshikimic acid was provided by Dr. G. Davies, Zeneca Pharmaceuticals. (6*R*)-6-Fluoroshikimic acid was converted enzymatically using shikimate kinase and EPSP synthase to (6*R*)-6-fluoro-EPSP as described by Balasubramanian et al. (1991). The progress of the enzymatic conversion was monitored using high-performance liquid chromatography (HPLC). The resulting reaction product was purified by means of HPLC using a Selectosil 5 Sax column (250 × 10 mm) from Phenomenex U.K. Ltd. under isocratic conditions with 1 M ammonium acetate (pH 6.2) as eluent and a flow rate of 1.5 mL min^{-1}. The product was then precipitated as the insoluble dibarium salt by addition of a 12-fold excess of barium acetate and three volumes of ethanol. After incubation at 4 °C for 12 h, the white precipitate was collected by centrifugation. The pellet was resuspended in 1 mL of distilled water and lyophilized. The dipotassium salt was prepared essentially as described for EPSP (Knowles et al., 1970). The concentration of the (6*R*)-6-fluoro-EPSP was determined using a modified molybdate/Malachite Green protocol using Triton N101 instead of Sterox detergent (Lanzetta et al., 1979) after dephosphorylation using alkaline phosphatase in Tris buffer (pH 8.4) containing 10 mM magnesium chloride.

Enzymes. Chorismate synthase was purified from an overproducing strain of *Escherichia coli* (AB2849/pGM605) as described recently (Bornemann et al., 1995a). From 20 g of cell paste, we typically obtained 125 mg of enzyme with a purity of >95% as judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−page). The enzyme was stored in liquid nitrogen.

Shikimate kinase and EPSP synthase were generously provided by Prof. J. Coggins, University of Glasgow, U.K. Xanthine oxidase was a generous gift of Prof. R. C. Bray of the School of Molecular Sciences, University of Sussex, Brighton, U.K. Alkaline phosphatase was purchased from Sigma Chemical Co., Poole, U.K.

UV−Vis Absorbance Spectrophotometry. Absorbance spectra were recorded with a Hewlett-Packard photodiode array instrument (model HP8452).

Sample Preparation for EPR and ENDOR Spectroscopy. Chorismate synthase stock solution (typically between 200 and 230 μM) was further concentrated to 1.0−1.2 mM using Centricon filters with a cutoff of 30 kDa. Stoichiometric amounts of FMN and (6*R*)-6-fluoro-EPSP were then added to the concentrated enzyme solution, and the final volume of the sample was brought to 250 μL by dilution with 50 mM MOPS buffer and 10% glycerol (pH 7.5). This solution was made anaerobic by repeated degassing and flushing with nitrogen and then transferred to a glovebox held under

nitrogen gas. Before the transfer to an appropriate sample tube, 0.25 μL of 1 M potassium oxalate was added to the enzyme solution. The flavinsemiquinone radical was generated in the sample tube by light irradiation (about 3−5 min; Ramjee et al., 1994) and the solution frozen immediately in the glovebox. The radical in the presence of chorismate was produced either by addition of a 3-fold excess of chorismate to the enzyme solution following the protocol described above or by reduction of the enzyme first to the fully reduced state and then addition of an 8-fold excess of EPSP anaerobically. In the latter case, EPSP is catalytically converted to chorismate. Further light irradiation of the sample is not required since in the presence of chorismate the fully reduced form of FMN is not stable and gives rise spontaneously to the neutral flavinsemiquinone in the presence of the catalytically generated chorismate. Samples in D_2O were prepared by repeated washing of the chorismate synthase solution with 50 mM MOPS buffer (pD 7.5) in D_2O in the presence of EPSP (or the fluoro analogue) and FMN in Centricon 30 microconcentrators.

Fluorescence Spectroscopy. Fluorescence emission spectra were recorded with a Kontron spectrofluorimeter (model SFM-25) equipped with a temperature-controlled cuvette holder. All fluorescence emission spectra reported here were recorded at 25 °C with an excitation wavelength of 365 nm.

Redox Titrations. The redox potential of oxidized FMN/reduced FMN in the presence of chorismate synthase was determined by the method recently described by Massey (1991). The cuvette contained 21.5 μM chorismate synthase, 17.2 μM FMN, 20 μM indigodisulfonic acid, 2 μM methyl viologen, and 500 μM xanthine in 800 μL of 50 mM MOPS buffer and 10% glycerol (pH 7) at 25 °C. After anaerobiosis was established, a small 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 concentrations of oxidized and reduced FMN as well as oxidized and reduced dye were 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 indigodisulfonic acid.

EPR and ENDOR Spectroscopy. Low-temperature EPR spectra were recorded with a standard Bruker ESP300 spectrometer operating at X-band frequencies which was equipped with a liquid helium Oxford Instruments ESR900 flow-cryostat. Continuous wave X-band ENDOR measurements were carried out with an updated Bruker ER 200D-SRC ENDOR/TRIPLE spectrometer with a Bruker TM_{110} cylindrical microwave cavity and the standard Bruker 16-turn rf coil. Radio frequencies were amplified using an ENI 100 W nominal power amplifier. The ENDOR spectrometer was likewise equipped with an Oxford Instruments ESR900 flow-cryostat. Suprasil quartz sample tubes with an outer diameter of about 5.0 mm were employed for maximum sample volume and ENDOR intensity.

ENDOR features, $\nu^{\pm}$, for an electron−nuclear hyperfine coupling, **A**, of an electron spin ($S = {}^1/_2$) with a proton spin ($I = {}^1/_2$) occur, when $\nu_n > |\mathbf{A}|/2$, in pairs symmetrically spaced around the nuclear Zeeman frequency $\nu_n = g_n\beta_n H_0/h$ (approximately 14.3 MHz at field settings close to $g = 2$ at X-band microwave frequencies), where g_n and β_n are the nuclear g factor and nuclear magneton, respectively, h is Planck's constant, and H_0 is the Zeeman field. The condition $\nu_n > |\mathbf{A}|/2$ is met for the proton couplings presented in the present work, so that the ENDOR transitions are expressed, to first order, by eq 1 with the two ENDOR frequencies given by

$$\nu^{\pm} = \nu_n \pm |\mathbf{A}|/2 \quad (1)$$

where **A** is the angular dependent hfc tensor. For a polycrystalline sample with a random distribution of the nuclear spin vectors with respect to the external magnetic field, for each of the two ENDOR resonances, $\nu^{\pm}$, we expect to obtain powder-type ENDOR spectra for an orientationally nonselective excitation.

RESULTS

Binding of Oxidized FMN to Chorismate Synthase. Addition of 12 μM chorismate synthase to a 6−10 μM FMN solution [50 mM MOPS buffer, containing 10% glycerol (pH 7.5)] had no affect on the UV−vis absorption spectrum of the flavin, in agreement with similar experiments reported earlier by Ramjee et al. (1993). In order to determine the K_D for oxidized FMN, these solutions were subjected to ultrafiltration (Centricon 30). Most of the flavin was found in the filtrate, allowing a K_D of *ca.* 30 ± 5 μM (three independent measurements) for oxidized FMN to be estimated. This result underlines the weak binding of oxidized FMN to chorismate synthase and is consistent with the observation that the flavin cofactor is entirely lost during purification of the enzyme, in particular during chromatography on cellulose phosphate where the enzyme is tightly bound to the column.

Addition of either EPSP, (6*R*)-6-fluoro-EPSP, or chorismate to an equimolar solution of oxidized FMN and chorismate synthase gives rise to spectral changes as shown in Figure 1A. All three ligands cause very similar effects with isosbestic points at 355, 405, 446, 458, and 470 nm resulting in a much more resolved UV−vis absorbance spectrum. Thus, the extinction coefficient at 444 nm remains almost unchanged compared to that of free oxidized FMN (12 500 M^{-1} cm^{-1}). As shown in Figure 1B, the spectral changes cease when the ternary complex with a 1:1:1 stoichiometry between the enzyme, oxidized flavin, and the ligand has formed. As a result of this tight binding, a K_D for the ligand cannot be obtained from these titrations. Ultrafiltration experiments carried out with oxidized FMN, chorismate synthase and EPSP, (6*R*)-6-fluoro-EPSP, or chorismate showed that oxidized FMN binds much more tightly to the enzyme in the presence of any one of the three ligands. The K_D from these experiments was calculated to be 20, 36, and 530 nM for EPSP, (6*R*)-6-fluoro-EPSP, and chorismate, respectively. This clearly demonstrates that flavin binding becomes considerably tighter in the presence of the substrate or its 6-fluoro analogue. The product chorismate, however, has a smaller effect on FMN binding.

Substrate or product binding is also associated with changes in the fluorescence emission spectrum of oxidized FMN. As shown in Figure 2, chorismate synthase causes a small decrease in the fluorescence emission intensity (18%), whereas upon addition of either EPSP or the fluoro analogue, the fluorescence emission maximum is shifted from 525 to 495 nm. This blue shift of the fluorescence peak is accompanied by a 23% decrease of the fluorescence intensity.

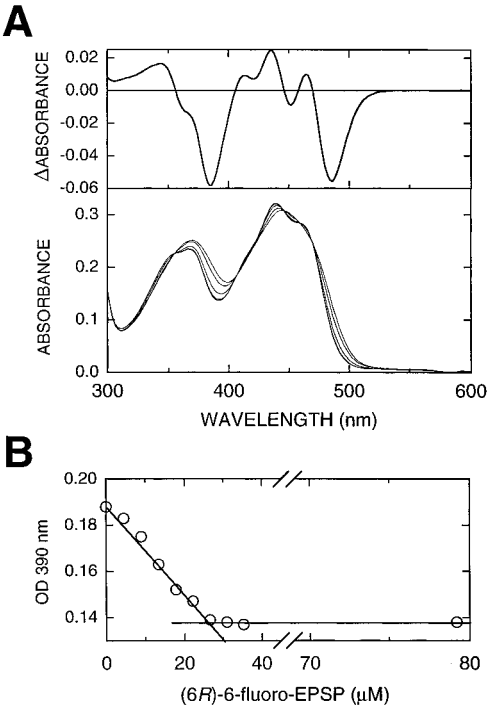


FIGURE 1: (A) UV−vis absorbance changes upon addition of (6*R*)-6-fluoro-EPSP to chorismate synthase and FMN. Chorismate synthase (26 μM) and FMN (23 μM) in MOPS buffer (50 mM) and 10% glycerol (pH 7.5) (total volume 800 μL) were titrated with (6*R*)-6-fluoro-EPSP at 25 °C. The spectra shown were recorded at the following (6*R*)-6-fluoro-EPSP concentrations: 0, 9, 17.8, 26.6, 35.3, and 79.2 μM. The spectral changes are characterized by isosbestic points at 355, 405, 446, 458, and 470 nm. The difference absorbance spectrum between the starting spectrum and the last spectrum at a (6*R*)-6-fluoro-EPSP concentration of 79.2 μM is shown at the top. (B) Shown are the absorbance changes at 390 nm as a function of the concentration of (6*R*)-6-fluoro-EPSP. The conditions are as detailed above.

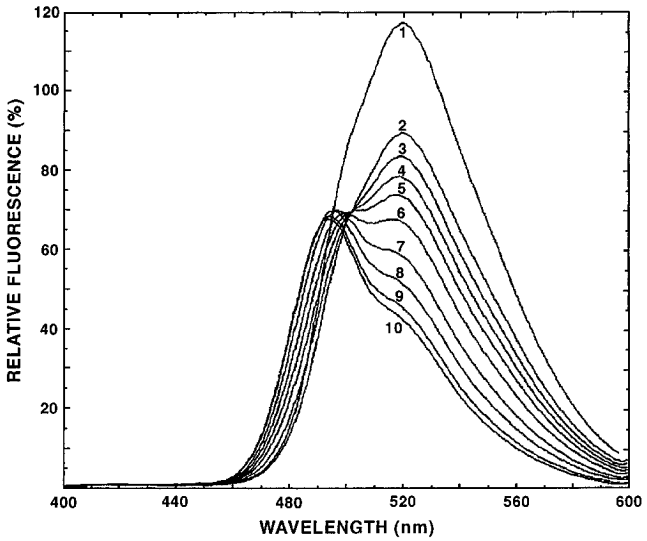


FIGURE 2: Fluorescence emission spectra of FMN in the presence of chorismate synthase upon addition of EPSP. Spectra were recorded in 50 mM MOPS buffer, 10% glycerol (pH 7.5) at 25 °C at an excitation wavelength of 365 nm. The first spectrum shown (trace 1) is that of free FMN (13.1 μM). Upon addition of chorismate synthase (14.3 μM), the fluorescence is slightly quenched (trace 2). Fluorescence emission spectra (traces 3−10) were recorded at EPSP concentrations of 2.64, 5.3, 7.9, 10.5, 13.2, 15.5, 18.5, and 34.3 μM, respectively.

An additional feature of the fluorescence spectrum in the presence of EPSP or (6*R*)-6-fluoro-EPSP is a shoulder at 520 nm. This shoulder is not due to free FMN since it was still present when the titration was carried out in the presence of a 50% excess of enzyme. After filtration of such a sample through a microconcentrator (Centricon 30), in order to remove any free flavin, the protein fraction had the same fluorescence features shown in Figure 2 (trace 10).

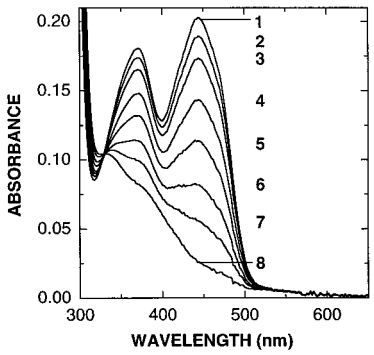


FIGURE 3: Reduction of FMN in the presence of chorismate synthase. FMN (17.2 μM) and chorismate synthase (21.5 μM) in 50 mM MOPS buffer and 10% glycerol (pH 7.5) containing 500 μM xanthine and 2 μM methyl viologen (800 μL) was made anaerobic by cycles of vacuum and flushing with nitrogen. After anaerobiosis was established, catalytic amounts of xanthine oxidase were added from a side arm of the cuvette. Reduction was monitored with time at 25 °C, and the spectra shown were recorded at the following times: before and 8, 18, 33, 48, 67, 88, and 124 min after the addition of xanthine oxidase.

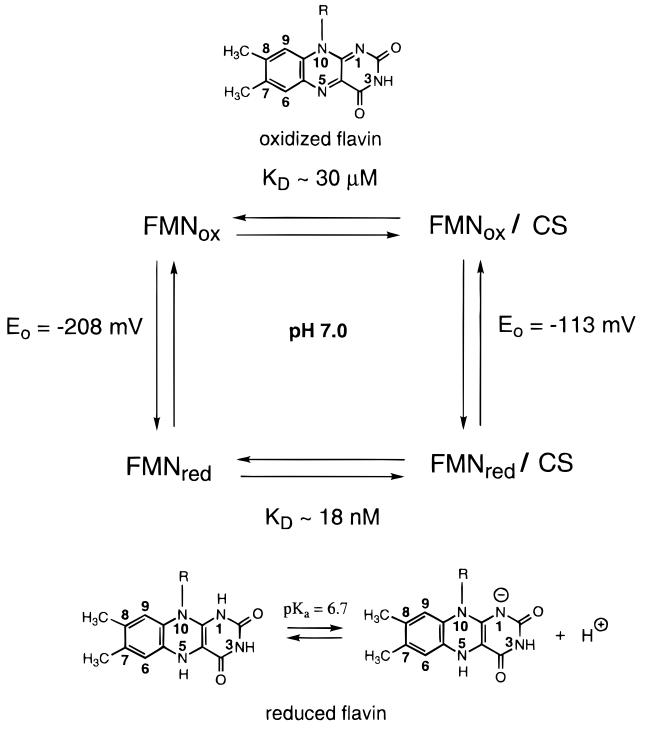
Chorismate, on the other hand, does not cause such a shift in the fluorescence maximum but gives rise to fluorescence quenching (25% residual fluorescence). Although chorismate yields perturbations of the oxidized flavin spectrum identical to those induced by the substrate and its fluoro analogue, its effect on the fluorescence emission spectrum is quite distinct.

Addition of sulfite (10 mM) to stoichiometric amounts of enzyme and FMN (10 μM) gave rise to an extremely slow formation of the sulfite−FMN adduct with a second order rate constant of approximately 0.8 ± 0.2 M^{-1} s^{-1}. The reaction, however, did not go to completion (80−90% adduct formation). In the presence of EPSP, this rate was much slower, and moreover, less sulfite−FMN adduct was formed, indicating that substrate binding further destabilizes formation of the sulfite−FMN adduct. Substrate binding may therefore inhibit the buildup of negative charge at the N(1)−C(2)═O locus of the isoalloxazine ring system that occurs upon sulfite−FMN adduct formation. Alternatively, this result could also be interpreted in terms of competitive binding of sulfite and EPSP.

Binding of Reduced Flavin to Chorismate Synthase. Reduction of FMN in the presence of chorismate synthase leads to fully reduced enzyme-bound flavin (Figure 3). The spectrum of reduced enzyme-bound FMN exhibits an absorbance maximum at 335 nm indicative of an N(1)-deprotonated flavin species. The course of reduction was independent of the reductant used, i.e. oxalate/light or an electron-generating enzymatic system (xanthine/xanthine oxidase). As can be clearly seen from Figure 3, no radical flavin species occurs during any stage of the reduction. Reoxidation of reduced FMN on shaking in air, although not studied quantitatively, was fast and occurred within the O_2 dissolution time (*ca.* 10 s). In the presence of stoichiometric concentrations of chorismate synthase, the redox potential (25 °C and pH 7) of the FMN/$FMNH_2$ couple is

shifted to −113 mV, indicating that the reduced form binds 1660 times tighter to the protein than does oxidized FMN (see Scheme 2). This amounts to a stabilization of the reduced form by 4.4 kcal/mol. The redox potentials of the oxidized FMN/reduced FMN couple free in solution and in the presence of chorismate synthase can be used to calculate the K_D for reduced FMN from the K_D determined for oxidized FMN as is depicted in Scheme 2.

Scheme 2



This thermodynamic relationship, correlating redox potentials and dissociation constants, allows a K_D for reduced FMN of *ca.* 18 nM to be calculated.

The redox potential of the oxidized FMN/reduced FMN couple was also measured as a function of pH. Between pH 6.3 and 8.8, the plot of the redox potential versus pH exhibits a slope of −30 mV per pH unit, indicating a two-electron/one-proton process. This is consistent with the observed UV−vis absorbance spectrum of the enzyme-bound reduced flavin species which bears features of the mono-anionic form, i.e. only one proton is bound to the flavin upon two-electron reduction.

Reduction of the Flavin in the Presence of EPSP, (6R)-6-Fluoro-EPSP, or Chorismate: Generation of the (Neutral) Flavinsemiquinone Radical. Reduction of FMN in the presence of the substrate analogue (6*R*)-6-fluoro-EPSP and chorismate synthase results in the generation of the neutral (blue) flavinsemiquinone (Figure 4). The spectral changes proceed with isosbestic points at 348, 386, 396, and 486 nm, and the final spectrum of the radical has absorbance maxima at 340, 392, 472, 552, and 584 nm ($\epsilon_{584\,nm} = 4700\ M^{-1}\ cm^{-1}$). Similarly, when FMN was reduced in the presence of the enzyme and EPSP or chorismate, radical formation was observed, although to a much lesser extent (40% with EPSP and 14% with chorismate on the basis of the extinction coefficient of 4700 $M^{-1}\ cm^{-1}$). Again, the extent of radical formation was independent of the mode of reduction. In the presence of EPSP and chorismate, reduction to the fully

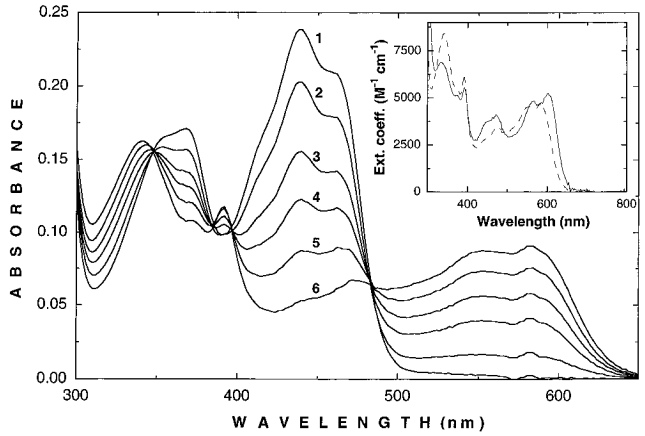


FIGURE 4: Photoreduction of FMN in the presence of chorismate synthase and (6*R*)-6-fluoro-EPSP. Chorismate synthase (21.5 μM), FMN (17.2 μM), and (6*R*)-6-fluoro-EPSP (106 μM) in 50 mM MOPS buffer and 10% glycerol (pH 7.5) (800 μL) was made anaerobic by repeated cycles of degassing and flushing with nitrogen. After anaerobiosis was established, 2 μL of a 1 M potassium oxalate solution was tipped in from a side arm of the cuvette. The first spectrum (trace 1) was recorded before light irradiation was started. The following spectra were recorded after 20 s and 1, 2, 4, and 10 min of light irradiation. The inset shows a comparison of the absorbance spectrum of the neutral flavin-semiquinone in the presence of (6*R*)-6-fluoro-EPSP (solid line) and chorismate (dashed line). The spectrum of the radical in the presence of (6*R*)-6-fluoro-EPSP shown is the last spectrum of the photo-reduction experiment (trace 6). The spectrum of the neutral flavinsemiquinone in the presence of chorismate was obtained by addition of EPSP (36 μM) to reduced flavin (15.7 μM) and chorismate synthase (17.2 μM). After conversion of EPSP to chorismate, the radical formed slowly and reached a maximum after 8 h (buffer and temperature as above).

reduced form occurred readily. The radical formed in the presence of the fluoro analogue, however, was difficult to reduce to the two-electron reduced flavin. Reoxidation of the radical with molecular oxygen was slow, with half-times ranging from several minutes in the presence of chorismate to *ca.* 1 h in the presence of the fluoro analogue.

The neutral flavinsemiquinone was still the predominant species at pH 9.5, suggesting that the pK_a associated with the formation of the anionic (red) flavinsemiquinone is significantly increased as compared with the pK_a of 8.3 observed for free flavin. The radical formed in the presence of (6*R*)-6-fluoro-EPSP or chorismate exhibits quite distinct spectral features as shown in the inset in Figure 4. The long wavelength absorbance maxima are shifted from 552 and 584 nm to 566 and 602 nm, respectively. The maxima at 472 and 392 nm, however, remained unchanged. In addition, the extinction coefficient for the radical in the presence of chorismate appears to be somewhat higher (ϵ_{max} at 602 nm $= 5200\ M^{-1}\ cm^{-1}$). The bathochromic shift observed with chorismate, compared with that of (6*R*)-6-fluoro-EPSP, indicates that the flavin experiences a more apolar environment in the presence of chorismate (Müller et al., 1972). The differences observed in the UV−vis spectrum are also reflected in the ENDOR spectra obtained with (6*R*)-6-fluoro-EPSP and chorismate as discussed in more detail below. The spectrum of the radical in the presence of chorismate, shown in the inset of Figure 4 (dashed line), was not obtained by reduction of the oxidized flavin species since this procedure, as mentioned above, gives a very low yield of the radical. On the other hand, if EPSP is added to an anaerobic sample of reduced FMN and chorismate synthase, EPSP is im-

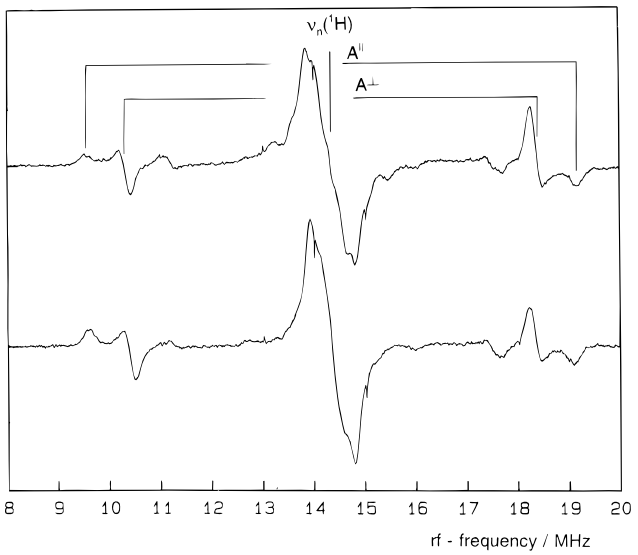


FIGURE 5: Frozen solution X-band ^{1}H-ENDOR spectrum of the flavinsemiquinone radical bound to chorismate synthase in the presence of (6*R*)-6-fluoro-EPSP. The external magnetic field was positioned at the center of the EPR line at $H_0 = 335.7$ mT. The ^{1}H-ENDOR spectrum (top spectrum) was recorded for the preparation in 50 mM MOPS buffer (H_2O, pH 7.5), and the other spectrum (bottom spectrum) was recorded using D_2O buffer. Enzyme concentrations were 0.62 mM (H_2O preparation) and 0.78 mM (D_2O preparation). The sharp spikes at integer rf values are spectrometer artifacts. Experimental conditions were as follows: temperature, 110 K; microwave frequency, 9.43 GHz (for the preparation in H_2O) and 9.46 GHz (for the preparation in D_2O); microwave power, 1 mW; modulation frequency, 12.5 kHz; and modulation depth, 88.9 kHz.

mediately converted to chorismate which is followed by the slow formation of the radical ($t_{1/2} \sim 1$ h). Obviously, the reduced flavin/enzyme complex is not stable under these conditions and is oxidized by an unknown agent to yield the flavinsemiquinone. Similarly, when (6*R*)-6-fluoro-EPSP was added to an anaerobic sample of chorismate synthase-bound, photoreduced FMN, rapid formation of the radical was observed (~50% of the total flavin). The formation of the radical was also observed when dithionite was used as reductant. Since excess dithionite did not prevent the formation of the radical, oxidation of reduced flavin to the flavinsemiquinone cannot be due to trace amounts of oxygen. Although we have not attempted to identify the chemical nature of the oxidant in this reaction, these observations indicate a low redox potential for the flavinsemiquinone/reduced FMN redox couple. Attempts to determine the redox potentials for the oxidized FMN/flavinsemiquinone and flavinsemiquinone/reduced FMN couples in the presence of (6*R*)-6-fluoro-EPSP using the method described by Massey (1991), however, were unsuccessful due to the nonideal behavior of the system. The slopes of the Nernst plots deviated from the expected value and moreover were found to vary with the dye used in the redox titrations.

EPR and ENDOR Spectroscopy. After either photoreduction or dithionite treatment, chorismate synthase stabilizes a radical which gives rise to an EPR signal at $g = 2.0039$ (neglecting a small g anisotropy) with a peak-to-peak line width of $\Delta H_{pp} \sim 2.1$ mT (independent of whether chorismate or (6*R*)-6-fluoro-EPSP was present). The EPR spectrum of the radical generated by photoreduction recorded at a temperature of 90 K is illustrated in the inset of Figure 6. The EPR spectrum only shows a single line with no resolved hyperfine structure. A similar unresolved EPR line was also observed for the radical in liquid solutions at room temperature (not shown), indicating that the rapid tumbling of the FMN radical is hindered by binding of FMN to an enzyme of high molecular weight (Carrington & McLachlan, 1967). Binding to tetrameric chorismate synthase with a relative molecular mass (M_r) ~156 000 will result in a rotational correlation time $\tau_R < 30$ ns (Kurreck et al., 1988), so that most of the dipolar hfcs are probably not averaged. Thus, even in solution at room temperature, the EPR spectrum for FMN bound to chorismate synthase is broadened by anisotropic contributions so that only a single unresolved line is observed.

The frozen solution X-band ENDOR spectrum of the chorismate synthase-bound flavinsemiquinone radical in the presence of the substrate analogue (6*R*)-6-fluoro-EPSP, recorded at the center position of the EPR spectrum, is shown in Figure 5. A number of pronounced ENDOR line pairs symmetrically spaced around the proton Larmor frequency, $\nu_n(^1H)$, are detected and hence are assigned to proton hfcs. The ^{1}H resonances with the largest ENDOR splittings (9.60 and 8.07 MHz) have been attributed, in accordance with other flavoproteins (Eriksson et al., 1970; Edmondson, 1985; Kurreck et al., 1984) to the two hf components $A^{\perp}$ and $A^{\parallel}$ of a tensor having axial symmetry from a freely rotating methyl group. The ENDOR line pair with the larger splitting of 9.60 MHz is the $A^{\parallel}$ component and the one with 8.07 MHz the $A^{\perp}$. These two values allow the determination of the isotropic contribution to the hf tensor from the expression $^1/_3(2A^{\perp} + A^{\parallel}) = A^{iso}$, yielding a value $A^{iso} = 8.58$ MHz. In addition, ENDOR line pairs from more weakly coupled protons were detected. The largest of these splittings was 6.3 MHz; similar values have been observed previously for semiquinones (Müller et al., 1970; Eriksson et al., 1970; Kurreck et al., 1984) and were attributed to a second class of protons of the semiquinone isoalloxazine ring.

In D_2O, the EPR line width decreases to $\Delta H_{pp} \sim 1.5$ mT (not shown). The comparison of the flavinsemiquinone ^{1}H-ENDOR spectrum in H_2O buffer (Figure 5, top) and the corresponding one in D_2O (Figure 5, bottom) shows that the spectra are very similar except for the region close to the ^{1}H matrix signal. This result suggests that some of the protons in the ternary complex with (6*R*)-6-fluoro-EPSP are exchangeable, but only those with relatively small couplings.

Figure 6 shows two different ^{1}H-ENDOR rf regions recorded under different experimental conditions. Figure 6A is the 16−20 MHz rf region of Figure 5 on an enhanced scale for the two EPR field settings I and II, shown on the EPR spectrum in the inset. The high-frequency components of the methyl proton signal can clearly be seen at about 18.4 and 19.2 MHz. With a field setting at position I, the ENDOR spectrum shows the typical powder-type line shape for each of the ENDOR line pairs of a hf tensor with axial symmetry arising from the coupling to methyl protons. In contrast, when field setting II was chosen, the high-frequency component, $A^{\parallel}$, of the powder spectrum is clearly absent and only the $A^{\perp}$ component remains. This demonstrates that for the FMN in chorismate synthase partial orientation selection in the ENDOR spectra is observable despite the small g anisotropy of the flavinsemiquinone radical which shows no perceptible asymmetry in the X-band EPR spectrum (inset of Figure 6).

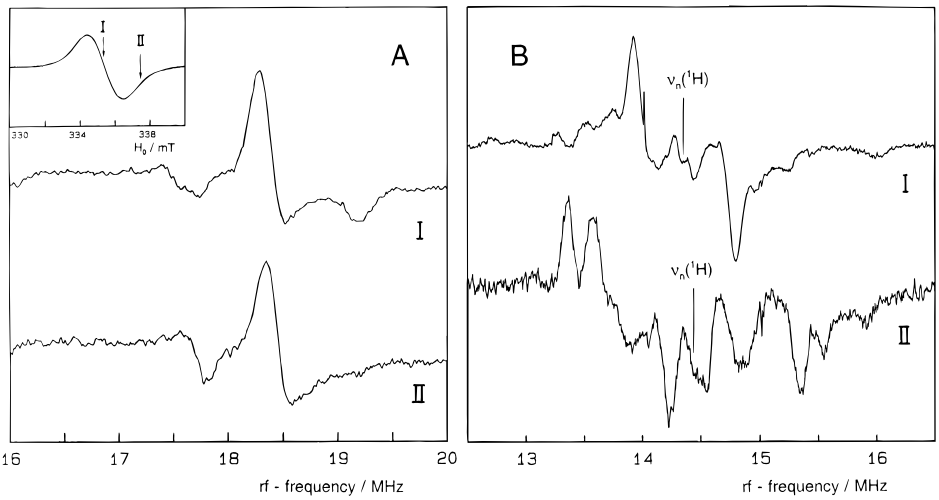


FIGURE 6: Frozen solution ^{1}H-ENDOR spectra of chorismate synthase in the presence of (6*R*)-6-fluoro-EPSP recorded at different temperatures and for two selected EPR field settings (spectra I and II in A and B, respectively). The EPR spectrum of the semiquinone radical is shown in the inset, and the respective field settings are indicated by I and II. Panel A shows the high-frequency set of ^{1}H-ENDOR resonances between 16 and 20 MHz on an expanded scale. Panel B is the central ^{1}H-ENDOR region recorded at a temperature of 200 K. The ^{1}H-ENDOR spectra were otherwise recorded for the same experimental conditions as those in Figure 5 except that for B the modulation depth was 50 kHz. The X-band EPR spectrum was recorded at a temperature of 90 K with 100 kHz modulation and 0.2 mW microwave power.

Figure 6B shows the central or matrix proton region on an expanded scale. It has previously been noted that, for flavin radicals, raising the temperature can cause the resolution of the region close to the matrix line to be significantly enhanced (Bretz, 1987). For this purpose, the ^{1}H-ENDOR spectrum was recorded at a sample temperature which was set to about 200 K. An ENDOR line width reduction is indeed observed, as Figure 6B illustrates, with the ENDOR line width decreased to about 150 kHz, compared with over 200 kHz at 110 K.

Experiments at higher temperatures were also performed in order to resolve possible interactions with a fluorine nuclear spin, $I = {}^1/_2$, which could arise from an interaction of the FMN radical with the fluorinated substrate analogue (6*R*)-6-fluoro-EPSP. The ^{19}F isotope has a nuclear Larmor frequency relatively close to that of protons (13.5 MHz at a magnetic field of ~336 mT), and thus, features from this nucleus would be asymmetrically spaced about the ^{1}H Larmor frequency at $\nu_n(^1H) = 14.3$ MHz. Assuming that the interaction is weak, the ^{19}F-ENDOR resonances would underlie the ^{1}H-ENDOR resonances close to the matrix region. However, no such resonances asymmetrically spaced about the ^{1}H Larmor frequency could be identified even in spectra recorded at ~200 K (cf. Figure 6B).

Figure 7A shows the comparison of the ^{1}H-ENDOR spectra, recorded over the full proton rf range, for the enzyme−flavinsemiquinone with bound substrate analogue (top) or product (bottom). Pronounced changes in the proton hf splittings are observed. This is best represented by the most prominent splittings from the methyl protons, as illustrated in Figure 7B, which shows the high-frequency region (as in Figure 7A) with the ENDOR line pair from the methyl group protons on an enlarged scale. The vertical lines indicate the shift of the splittings depending on whether the fluoro analogue or chorismate is bound. The hfcs in the presence of chorismate, of $A^{\perp} = 7.56$ MHz and $A^{\parallel} = 9.08$ MHz, are both about 0.5 MHz smaller than the respective ones with (6*R*)-6-fluoro-EPSP. A similar observation was made for some of the other ^{1}H-ENDOR resonances. All ^{1}H-ENDOR hf splittings are listed in Table 1. This clearly

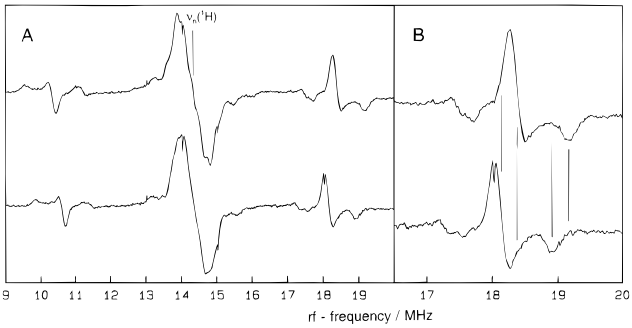


FIGURE 7: Comparison of the frozen solution ^{1}H-ENDOR spectra of the semiquinone radical in the presence of (6*R*)-6-fluoro-EPSP (top spectrum) and chorismate (bottom spectrum). The complete ^{1}H-ENDOR spectrum is shown in A. The high-frequency part of the spectrum is depicted again in an expanded scale in B. The vertical lines in B illustrate the shift of the two hyperfine components, $A^{\parallel}$ and $A^{\perp}$, depending on the presence of either (6*R*)-6-fluoro-EPSP or chorismate. Spectrometer conditions were those used for Figure 5.

indicates that the substrate/product has a critical influence, not only on the ^{1}H-ENDOR line pairs arising from the methyl protons, reported above, but also affects interactions with other protons.

The product-bound form was also studied after formation of chorismate from EPSP. As Table 1 demonstrates, no significant differences have been detected in the hf parameters of the semiquinone radical depending on whether chorismate was added directly or by enzymatic generation from EPSP. Spectra were also recorded for the chorismate-containing sample in D_2O buffer solution at both 110 K and about 200 K (data not shown), showing a substantial line width reduction at higher temperatures similar to that with the fluoro analogue.

ENDOR resonances for the enzyme in the presence of (6*R*)-6-fluoro-EPSP at lower radio frequencies (data not shown), which do not arise from proton hf interactions, have also been detected. These resonances show a single line centered at a frequency of about 5.8 MHz (with a field setting at $H_0 = 335.7$ mT). This resonance is most probably due to an interaction of the radical spin with a phosphorus nuclear

Table 1: Proton hfc Constants in Megahertz for the FMN Radical in the Enzyme−FMN−Substrate/Product Complex of Chorismate Synthase (CS)[a]

CS + FMN + (6*R*)-6-fluoro-EPSP			CS + FMN + chorismate[b]			CS + FMN + chorismate[c]			
$A^{\perp}$	$A^{\parallel}$	A^{iso}	$A^{\perp}$	$A^{\parallel}$	A^{iso}	$A^{\perp}$	$A^{\parallel}$	A^{iso}	assignment
8.07	9.60	8.58	7.56	9.08	8.07	7.56	9.09	8.07	$CH_3(8\beta)$
	6.3			5.8			6.0		H(6α)
	3.30[d]			–			–		unassigned
	2.16[d]			2.3			2.4		unassigned
	1.63[d]			1.7			1.8		$[CH_3(7\beta)]$[e]
	1.15[d]			0.9			1.05[d]		unassigned
	0.72[d]			-			0.73[d]		unassigned
	0.51[d]			0.4			0.53[d]		unassigned

[a] Values below the dashed line represent observed hf splittings which have not been assigned to specific hf tensor components. Estimated errors are ±0.05 MHz. The unassigned protons can arise either from small couplings of protons of the flavinsemiquinone or from the protein environment. [b] Prepared by direct addition of chorismate. [c] Chorismate formed from EPSP. [d] Determined from spectra recorded at ~200 K. [e] The assignment of this ENDOR splitting to the methyl (7β) protons is not unambiguous (see text).

spin $I = {}^1/_2$ from natural abundance ^{31}P, which at this field strength has a nuclear Larmor frequency of $\nu_n = 5.79$ MHz. The observation of only a phosphorus matrix line, with no splitting, indicates that the interaction with ^{31}P is weak. Similar ENDOR experiments have been carried out with chorismate (which has no phosphate group) bound to chorismate synthase, and a similar phosphorus matrix ENDOR signal from weakly coupled ^{31}P nuclei was observed.

DISCUSSION

Addition of the substrate EPSP, (6*R*)-6-fluoro-EPSP, or the product chorismate leads to a better resolved UV−vis absorbtion spectrum of the flavin. The spectral perturbations observed are very similar to the solvent effects described earlier by Müller et al. (1973), suggesting that the flavin binding pocket of chorismate synthase provides an apolar environment. In this ternary complex between enzyme, FMN, and ligand (i.e. any of the three compounds mentioned above), oxidized flavin is much more tightly bound; depending on the ligand used, binding is between 55- and 1500-fold tighter. Titrations performed with any of the three ligands (see Figure 1) yielded a sharp end point indicating that a 1:1:1 ternary complex is formed between enzyme, oxidized FMN, and ligand.

Binding of the oxidized flavin in the presence of either EPSP or the (6*R*)-6-fluoro-EPSP is also associated with a shift of the fluorescence emission maximum from 525 nm (free oxidized flavin) to 495 nm. This hypsochromic shift can be interpreted in terms of either a more hydrophobic flavin environment or a restrained flavin mobility upon binding to the apo-protein. The first explanation, however, seems unlikely since the difference of the dipole moment of the flavin in its ground (S_o) and first excited state (S_1) is only 1 D (Shcherbatska et al., 1992) and therefore cannot account for the observed hypsochromic shift of the fluorescence emission maximum. Thus, restrained mobility of the bound oxidized flavin must be largely responsible for the observed shift of the fluorescence emission maximum. Interestingly, chorismate itself does not lead to restrained mobility. The only effect of chorismate is to quench the flavin fluorescence.

In contrast to oxidized flavin, reduced flavin binds tightly to the enzyme in its anionic form. When free in solution, reduced FMN exhibits a pK_a of 6.7 for the proton bound to N(1). A characteristic feature of the anionic species is an absorbance maximum around 345 nm, and this was observed for chorismate synthase-bound reduced FMN even at a pH of 6.2. This indicates that the N(1) proton of enzyme-bound reduced flavin has a significantly lower pK_a than that of free reduced FMN. Corroborating evidence is provided by the pH dependence of the redox potential of the oxidized FMN/reduced FMN couple which exhibits a slope of −30 mV per pH unit, clearly indicating that the two-electron reduction is accompanied by a single proton uptake. Preferential binding of reduced flavin over the oxidized form has also been found for bacterial luciferase where binding of reduced FMN is approximately 100-fold tighter (Meighen & Hastings, 1971; Baldwin et al., 1975). Unlike chorismate synthase, however, reduced flavin bound to bacterial luciferase reacts with oxygen to yield a flavin−4a-hydroperoxide intermediate which then reacts with a long chain aldehyde, such as decanal, to give rise to the corresponding fatty acid, water, and light (Hastings et al., 1973). Hence, despite their similar requirements for reduced flavin and their higher affinities for the reduced form of FMN, both enzymes catalyze very different reactions.

Photoreduction of FMN bound to chorismate synthase in the presence of (6*R*)-6-fluoro-EPSP leads to complete formation of the neutral (blue) flavinsemiquinone. Since this radical was stable toward disproportionation in the presence of electron mediators such as methyl or benzyl viologen, the stabilization of the radical species is clearly thermodynamic, rather than kinetic, assuming communication between the flavin and the mediator. Stabilization of a neutral flavinsemiquinone by the apo-protein can be achieved by a strong hydrogen bond to the N(5) proton. This was in fact observed for flavodoxins where the three-dimensional structure confirmed the existence of a hydrogen bond between the N(5) proton and a carbonyl group (Smith et al., 1977). Alternatively, an apolar flavin binding site would also stabilize the neutral radical species more than the anionic species. The pK_a of 8.3 for the free radical is certainly increased to a value greater than 10 since chorismate synthase was found to bind the neutral form even at pH 9.5. Additional evidence for preferential binding of neutral over negatively charged species is provided by experiments using sulfite as a reagent to form an N(5)−flavin adduct. The reaction with sulfite was very slow in the absence of EPSP and even slower in its presence. These results suggest that chorismate synthase binds sulfite only very weakly and hence

Table 2: Isotropic Hyperfine-Coupling Constants for the $CH_3(8\beta)$ Protons of the Semiquinone Obtained for Neutral FMN Radicals ($FMN^{\bullet}$) of Some Flavin-Binding Proteins Compared with the hf Coupling for the FMN Radical ($FMN^{\bullet-}$) of the Anionic Form from Methanol Oxidase

protein	A^{iso} (MHz)	reference
flavodoxin from *Megasphaera elsdenii*	8.5	Kurreck et al., 1984
flavodoxin from *Azotobacter vinelandii*	8.3	Kurreck et al., 1984
flavodoxin from *Anabaena* PCC 7119	8.22	Medina et al., 1995
ferredoxin−$NADP^+$ reductase	8.12	Medina et al., 1995
chorismate synthase + (6*R*)-fluoro-EPSP	8.58	this work
chorismate synthase + chorismate	8.07	this work
methanol oxidase from *Candida boidinii*	10.6	Kurreck et al., 1984

does not stabilize a negative charge at the N(1)−C(2)═O locus, in contrast to flavoprotein oxidases. In summary, our results demonstrate that chorismate synthase provides a rather apolar environment for the flavin in the presence of EPSP, (6*R*)-6-fluoro-EPSP, or chorismate. Since reduced FMN is bound in its monoanionic form to chorismate synthase in the absence of these ligands, binding of the substrate to the reduced FMN−enzyme complex is most probably accompanied by protonation of the N(1) position.

The line width of the EPR signal (∼2.1 mT) as well as the ^{1}H-ENDOR splitting for the methyl group proton hf tensor components (Table 2) are characteristic of the neutral flavinsemiquinone radical. This is consistent with the results derived from optical spectroscopy.

The largest ENDOR splittings have been attributed, in accordance with the assignment for other flavoproteins (Eriksson et al., 1970), to the two hf components from protons of the freely rotating methyl group at the 8-position of the flavin cofactor (for numbering see Scheme 2). In addition to the methyl group protons, other ^{1}H couplings were detected. The ^{1}H-ENDOR line pair with the next smaller splitting was observed with a hf component of about 6 MHz depending on whether the fluoro analogue or chorismate was bound. This splitting is considerably larger than the couplings reported for the α-proton at position 6 of the isoalloxazine ring in other protein-bound neutral flavinsemiquinones with 5.5−5.6 MHz for flavodoxins and 5.2−5.6 MHz for some flavin model compounds (Kurreck et al., 1984) irrespective of whether substrate or product is bound. However, we nonetheless tentatively attribute this ^{1}H-ENDOR line pair to one hf component arising from the 6α-proton since powder ENDOR hf splittings from other protons, except the C(8)-methyl protons, are significantly smaller. Hyperfine couplings to α-protons usually give rise to rhombic ENDOR spectra (McConnell et al., 1960). Interactions with a high anisotropy are often of low intensity, as observed for other organic radicals (O'Malley & Babcock, 1986), so that in our case for the rhombic 6α-proton spectrum, not all ENDOR resonances could be identified. Thus, the hf tensor is incomplete and the isotropic hfc constant for the α-proton cannot be calculated. However, if the observed resonance is the central crossing peak of the hf pattern and the other resonances are symmetric about each of the central features, the measured splitting of 6.3 MHz would equal A^{iso}. Nonetheless, this value is only an approximation to the α-proton hfc.

One of the small ENDOR splittings with a splitting of 1.8 MHz could be due to the second methyl protons of the flavinsemiquinone at the 7-position (put in square brackets in Table 1). For these methyl protons, splittings of about 1.6 MHz are reported for other flavins (Kurreck et al., 1984). However, due to the number of ^{1}H resonances with similar splittings, an unequivocal assignment cannot be made.

The ENDOR hf parameter of the $CH_3(8\beta)$ protons exhibits a pronounced shift depending on whether the substrate analogue or the product is bound to chorismate synthase. The isotropic hfcs of the methyl(8β) protons found for chorismate synthase in the presence of either (6*R*)-6-fluoro-EPSP or chorismate are compared, in Table 2, to those measured for other protein-bound FMN radicals. In fact, the changes observed for chorismate synthase are such that A^{iso} for the radical with (6*R*)-6-fluoro-EPSP exceeds most of the values found previously for other neutral flavinsemiquinone radicals in FMN binding proteins. Conversely, the value with chorismate is considerably smaller than the respective values for other proteins (cf. Table 2). From the observed shift of the hf splitting, it is suggested that the flavin $CH_3(8\beta)$ protons sense whether the substrate analogue or the product is bound. This implies that the spin density distribution at this position undergoes quite significant rearrangements when the substrate analogue is replaced by the product.

Shifts of the hfcs, i.e. a larger value for the substrate analogue compared to the product, were also observed for the splitting, which has been tentatively attributed to a coupling to the α-proton at position 6, demonstrating not only that the hfc to the $CH_3(8\beta)$ protons does change but also that interactions with protons at other positions in the benzene moiety of the isoalloxazine are also affected. We therefore conclude that the substrate/product binding site is closely associated with FMN and is able to communicate with the semiquinone radical even though no direct interactions with nuclei of the substrate have been identified by ENDOR.

Very minor changes in the ^{1}H-ENDOR spectra are observed for preparations in H_2O and D_2O buffer solution. This indicates that relatively few protons are susceptible to exchange. This is consistent with a relatively hydrophobic environment for the radical with few exchangeable hydroxyl protons in the immediate radical environment. A similarly hydrophobic environment has been indicated for other flavoenzymes, such as "old yellow enzyme" (Kurreck et al., 1984).

Using (6*R*)-6-fluoro-EPSP, we have also attempted to detect interactions between the flavinsemiquinone radical and nuclear spins from other nuclei such as ^{31}P and ^{19}F. No couplings could be assigned to an interaction of the radical spin with ^{19}F of the substrate analogue, suggesting that an electron−nuclear superhyperfine interaction with ^{19}F is too weak to be detected. However, due to the very similar nuclear Larmor frequencies of ^{1}H and ^{19}F, it is possible that the fluorine interactions are obscured by the rather large matrix proton peak. On the other hand, a weak interaction of the radical with a phosphorus nucleus has been observed. Such a weak phosphorus coupling could potentially arise from an interaction with the phosphate group from the substrate analogue. However, since a very similar phosphorus matrix peak was also detected with the chorismate, which does not have a phosphate group, it is unlikely that the ENDOR signal arises from the phosphate group of the

(6*R*)-6-fluoro-EPSP and more likely results from an interaction with the FMN ribityl phosphate group or adventitious phosphate.

Since the neutral flavinsemiquinone described here forms upon reduction of flavin in the presence of chorismate synthase and EPSP, it appears likely that *in vivo* the flavin is reduced prior to the formation of the ternary substrate−FMN−enzyme complex. The flavinsemiquinone was not observed during turnover with EPSP (Ramjee et al., 1991) or (6*S*)-6-fluoro-EPSP (Bornemann et al., 1995b). However, with both of these substrates, the flavinsemiquinone radical forms slowly after complete consumption of the substrate. Moreover, addition of (6*R*)-6-fluoro-EPSP to enzyme-bound reduced FMN results in the rapid formation of the flavinsemiquinone (Ramjee et al., 1992). These observations suggest that the enzyme-bound reduced FMN has a very low redox potential which allows the flavin to reduce another, as yet unidentified, compound. However, Ramjee et al. (1992) ruled out the presence of transition metal ions and covalently attached cofactors using plasma emission spectroscopy and electrospray mass spectrometry, respectively.

A low redox potential for the flavinsemiquinone/reduced FMN couple is a characteristic of flavodoxins. In fact, chorismate synthase shares some other salient features with flavodoxins, such as the preferential binding of neutral flavin species in the presence of substrate or product. On the basis of these similarities between flavodoxin and chorismate synthase, it is tempting to speculate that chorismate synthase-bound reduced FMN serves as an electron donor to the substrate leading to cleavage of the C−O bond (Bornemann et al., 1995b). The catalytic cycle is then completed by homolytic C−H bond cleavage and back-transfer of an electron to the flavinsemiquinone. Alternatively, the electron-rich reduced FMN could stabilize an intermediate allylic carbocation resulting from the initial phosphate cleavage proposed by Bartlett et al. (1989) and Hawkes et al. (1990).

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