The Transient Kinetics of *Escherichia coli* Chorismate Synthase: Substrate Consumption, Product Formation, Phosphate Dissociation, and Characterization of a Flavin Intermediate†

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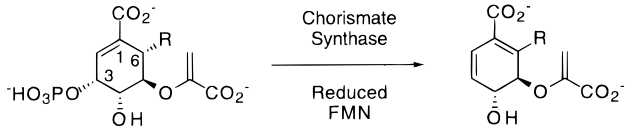
ABSTRACT: Chorismate synthase is the seventh enzyme of the shikimate pathway and catalyzes the conversion of 5-enolpyruvylshikimate 3-phosphate (EPSP) to chorismate. The reaction involves the 1,4-elimination of phosphate and the C-(6*proR*) hydrogen of the substrate with unusual *anti* stereochemistry and requires a reduced flavin cofactor. This paper describes the kinetics of the formation and decay of a flavin intermediate, EPSP consumption, chorismate and phosphate formation, and phosphate dissociation during single and multiple turnover experiments, determined using rapid reaction techniques. The kinetics of phosphate dissociation using the substrate analogues (6*R*)-[6-^{2}H]EPSP and (6*S*)-6-fluoro-EPSP have also been determined. The observations are consistent with a nonconcerted chorismate synthase reaction. The flavin intermediate is not simply associated with the conversion of substrate to product because it forms before the substrate is consumed. The transient spectral changes must be associated primarily with events such as protonation of the reduced flavin, a charge transfer complex between reduced flavin and an aromatic amino acid, or a conformational change in the protein. This does not rule out the direct role of flavin in catalysis.

Chorismate synthase (EC 4.6.1.4) is the seventh enzyme of the shikimate pathway (Bentley, 1990; Haslam, 1993) and catalyzes the conversion of 5-enolpyruvylshikimate 3-phosphate (**1**) (EPSP)[1] to chorismate (**2**) (Scheme 1). Chorismate synthase catalyzes a 1,4-elimination of phosphate and the C-(6*proR*) hydrogen from EPSP with overall *anti* stereochemistry (Hill & Newkome, 1969; Onderka & Floss, 1969; Floss et al., 1972). Model systems (Hill & Bock, 1978; Toromanoff, 1980) and molecular orbital calculations (Fukui, 1965; Anh, 1968) suggest that concerted 1,4-elimination reactions favor *syn* stereochemistry. For these reasons, nonconcerted mechanisms have been suggested for the chorismate synthase reaction (Walsh et al., 1990; Bornemann et al., 1996). Kinetic isotope effect studies using the *Neurospora crassa* enzyme are consistent with a nonconcerted mechanism (Balasubramanian et al., 1995). An X-group mechanism, involving nucleophilic attack at the C(1) position of EPSP (Floss et al., 1972), appears to be unlikely (Hawkes et al., 1990; Bornemann et al., 1996). Studies with (6*S*)-6-fluoro-EPSP (**3**) (Scheme 1) (Bornemann et al., 1995a) and (6*R*)-[6-^{2}H]EPSP (Bornemann et al., 1995b) suggest that a mechanism involving the initial deprotonation at C(6) to form an anionic allylic intermediate is also unlikely. An allylic rearrangement of phosphate followed by a 1,2-elimination (Ganem, 1978) has also been discounted (Bartlett et al., 1986).

Scheme 1



1, R = H
3, R = F

2, R = H
4, R = F

None of the above mechanisms take into account the role of the flavin cofactor of chorismate synthase. Reduced flavin mononucleotide (FMN) is required for enzyme activity despite there being no overall redox change in the conversion of EPSP to chorismate (Morell et al., 1967; Welch et al., 1974; White et al., 1988; Ramjee et al., 1993; Schaller et al., 1991). Strong evidence that reduced flavin is chemically, and not just structurally, involved in catalysis comes from the lack of detectable activity of the *N. crassa* (Lauhon & Bartlett, 1994) and *Escherichia coli* (Bornemann et al., 1995c) enzymes reconstituted with reduced 5-deaza-FMN. Additional evidence comes from the observation of a transient flavin intermediate that has been characterized by UV/visible spectrophotometry during single and multiple turnover experiments with the enzyme from *E. coli* (Ramjee et al., 1991; Bornemann et al., 1994). It was suggested that the spectrum of this flavin intermediate was consistent with either a C(4a)-flavin adduct or a charge transfer complex. Furthermore, the formation of a protein-bound neutral flavin semiquinone in the presence of the inhibitor (6*R*)-6-fluoro-EPSP with the *E. coli* enzyme suggests the possibility of radical intermediates during normal turnover (Ramjee et al., 1992). The absence of an obvious hydrogen atom acceptor rules out the possibility of a proposed radical mechanism (Bartlett et al., 1989; Lauhon & Bartlett, 1994; Giese & Almstead, 1994) involving the initial removal of a hydrogen atom from the C(6*proR*) position of EPSP. A more likely

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[1] Abbreviations: EPSP, 5-enolpyruvylshikimate 3-phosphate; FMN, riboflavin 5′-phosphate mononucleotide; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; HPLC, high-performance liquid chromatography; PBP, phosphate binding protein.

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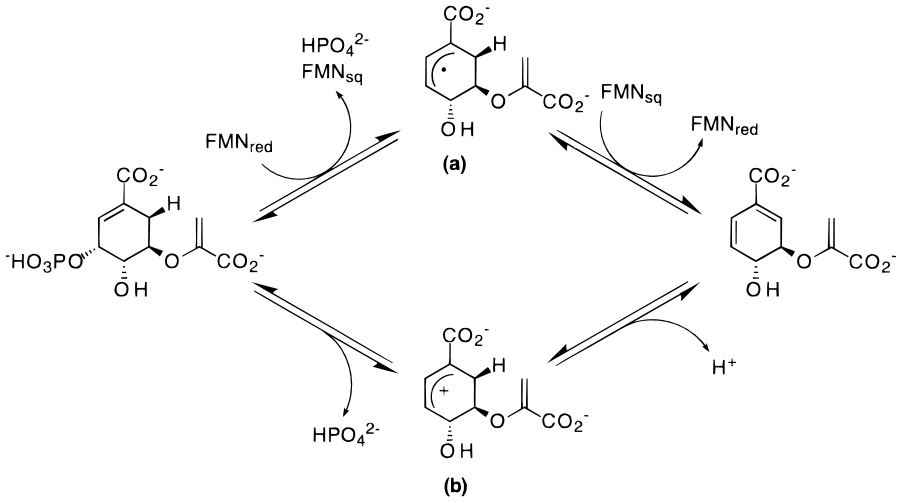
Scheme 2



radical mechanism (Scheme 2a) would involve the donation of an electron from the reduced flavin to EPSP, leading to the elimination of phosphate (Bornemann et al., 1995a). The abstraction of a hydrogen atom from the allylic radical intermediate by the resultant flavin semiquinone would give the product chorismate and regenerate the reduced flavin cofactor. Studies with the substrate analogue (6*S*)-6-fluoro-EPSP (Bornemann et al., 1995a) suggest that this radical mechanism and a cationic mechanism (Bartlett et al., 1989; Hawkes et al., 1990), involving the stepwise loss of phosphate and a proton (Scheme 2b), are the most likely. Although there is no obvious mechanistic role for the cofactor in the cation mechanism, reduced flavin is electron rich and could play a role in the stabilization of the cationic intermediate. Both of these mechanisms involve the ordered cleavage of the C(3)−O and C(6*proR*)−H bonds in discrete steps. Single turnover experiments with (6*R*)-[6-^{2}H]EPSP have shown that the flavin intermediate forms before the C(6*proR*)−H bond is cleaved (Bornemann et al., 1995b). To help identify the reaction mechanism, it is important to establish the order of C(3)−O cleavage and the formation of the flavin intermediate.

This paper describes the kinetics of the formation and decay of the flavin intermediate, EPSP consumption, product formation, and phosphate dissociation during single and multiple turnover experiments using EPSP and substrate analogues. The results show that the flavin intermediate forms before either of the C(3)−O and C(6*proR*)−H bonds are cleaved and suggest that the reaction is nonconcerted. The implications for the reaction mechanism and the nature of the flavin intermediate are discussed.

MATERIALS AND METHODS

Materials. All chemicals and biochemicals were of the highest grade available and unless otherwise stated were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Sodium dithionite was purchased from B. D. H. Chemicals (Poole, Dorset, U.K.). The potassium salts of EPSP (Knowles et al., 1970) and (6*R*)-[6-^{2}H]EPSP (Balasubramanian & Abell, 1991; 80% isotopic purity, the remainder being in the protio form) were prepared as described previously (Bornemann et al., 1995b). (6*S*)-6-Fluoroshikimic acid (Sutherland et al., 1989) was provided by ZENECA Pharmaceuticals and was converted to (6*S*)-6-fluoro-EPSP as described previously (Balasubramanian et al., 1991) with modifications (Bornemann et al., 1995a).

Methods. All experiments were performed at 25 °C, and solutions were buffered using 50 mM MOPS/KOH, pH 7.5, unless otherwise stated. All spectrophotometric measurements were obtained with a 1.0 cm path length. Quoted errors are sample standard deviations (HP 32S II).

Enzyme Purification. Recombinant *E. coli* chorismate synthase (subunit M_r = 39 000: White et al., 1988; Ramjee et al., 1993) was purified as reported previously (Bornemann et al., 1995b) to a specific activity of 27.4 μmol of chorismate produced min^{-1} mg^{-1}. Protein concentration was estimated using ϵ_{280} = 21 440 M^{-1} cm^{-1} per subunit (Mach et al., 1992). The enzyme was stored in the presence of MOPS buffer and 20% glycerol in liquid nitrogen for over 1 year without any detectable loss of activity. Glycerol was added to substrate solutions to match the final glycerol concentration in enzyme solutions used for stopped-flow spectrophotometry/fluorometry and rapid chemical quench experiments to avoid inefficient mixing. Adventitious inorganic phosphate was removed from purified enzyme by elution from a desalting column using MOPS buffer containing 20% glycerol and 50 mM K_2SO_4. The K_2SO_4 was required to maintain the solubility of the enzyme (in the absence of substrate and FMN) at high protein concentrations throughout this procedure. The addition of 50 mM K_2SO_4 had no effect on the steady-state and transient kinetics of the enzyme observed by spectrophotometry. This observation also shows that the enzyme is insensitive to such changes in ionic strength.

Spectrophotometric Enzyme Assays. The formation of the product diene (chorismate ϵ_{275} = 2630 M^{-1} cm^{-1}) was monitored as previously described (Ramjee et al., 1994), and substrate concentrations were determined using this method. Anaerobic assay mixtures contained substrate (50 μM), FMN (10 μM), potassium oxalate (1 mM), and buffer. The FMN was photoreduced, with oxalate being the electron source, before the addition of enzyme giving a final volume of 500 μL.

Spectra of Flavoenzyme in the Oxidized, Semiquinone, and Reduced States. The oxidized holoenzyme was obtained by mixing stoichiometric amounts of chorismate synthase, oxidized FMN, and the substrate EPSP (Macheroux et al., 1996). The semiquinone state was obtained using (6*R*)-6-

fluoro-EPSP as described previously (Ramjee et al., 1992; Macheroux et al., 1996). The reduced state was obtained by photoreducing a stoichiometric mixture of chorismate synthase and FMN under anaerobic conditions in the presence of potassium oxalate (1 mM). UV/visible spectra were obtained using a Shimadzu MPS-2000.

Stopped-Flow Spectrophotometry and Spectrofluorometry. Single turnover experiments were carried out using a Hi-Tech Scientific SF-51 stopped-flow spectrophotometer/fluorometer (Salisbury, U.K.) modified, installed, and operated in an anaerobic glovebox as described previously (Thorneley & Lowe, 1983). The tubing of the sample handling unit was modified in order to minimize its gas permeability. The dead time of the instrument was measured to be 4.1 ± 0.3 ms (Ramjee, 1992), and all data are corrected for a 4 ms dead time. The stopped-flow observation cell had a 1.0 cm path length. Anaerobic substrate solution was mixed with anaerobic enzyme solution containing $FMNH_2$ that was reduced using either dithionite or photoirradiation in the presence of potassium oxalate. Spectra of reaction intermediates were obtained using a Hi-Tech Scientific MG-6000 rapid scanning photodiode array (standard configuration with 512 diodes) coupled to the SF-51 stopped-flow sample handling unit. Difference spectra were obtained using a control experiment without substrate as the blank. Data scanned between 350 and 700 nm were collected with the minimum integration time allowed by the instrument of 1.25 ms. The Xe light source used for the rapid scanning experiments emits high intensity light between 350 and 800 nm and substantially lower intensity light below 350 nm. The introduction of a Hi-Tech UG5 filter between the Xe light source and the sample handling unit was necessary when scanning between 280 and 560 nm in order to obtain a good signal to noise ratio for data below 350 nm. The filter reduced the intensity of light above 400 nm, enabling the transmission in the appropriate wavelength range to be maximal. An increased integration time of 5 ms was also required to compensate for the relatively low light intensity used for these scanning experiments. Kinetic data were fitted using the Hi-Tech Scientific IS-2 v2.3b5 software.

Rapid Chemical Quench. Rapid chemical quench experiments were carried out using the KinTek RQF3 with nominally 40 μL reactant loops with 1 M $HClO_4$ as the quenching agent. A number of precautions were taken in order to ensure the anaerobicity of the reactants during each experiment: (1) the oxygen concentration in the thermostating water was reduced to <3 μM, to reduce the risk of oxygen diffusing through plastic tubing and connectors, by sparging with nitrogen gas for at least 3 h prior to use; (2) the drive solutions were anaerobic MOPS buffer containing 1 mM sodium dithionite; (3) the reactant and reaction loops were thoroughly flushed with nitrogen gas before each experiment. After each experiment, the precipitated protein in the samples was removed using an Eppendorf centrifuge at 4 °C for 10 min. A zero time-point sample was prepared by mixing appropriate volumes of enzyme solution (42.7 μL) and drive buffer (25 μL) with acid quench (180 μL) and then adding substrate solution (38.9 μL) last. The samples were analyzed either by HPLC for substrate and product or by spectrophotometric methods for inorganic phosphate.

HPLC. Compounds were separated using a Phenomenex U. K. Ltd. Selectosil 5 SAX semipreparative column (Macclesfield, U.K.; 250 × 10 mm) fitted with a guard column (50 × 10 mm) with an isocratic eluant of 1 M ammonium acetate, pH 6.2. EPSP (retention time 16.0 min) and chorismate (25.0) were eluted at a flow rate of 1.5 mL min^{-1} and monitored by their UV absorbance at 230 nm.

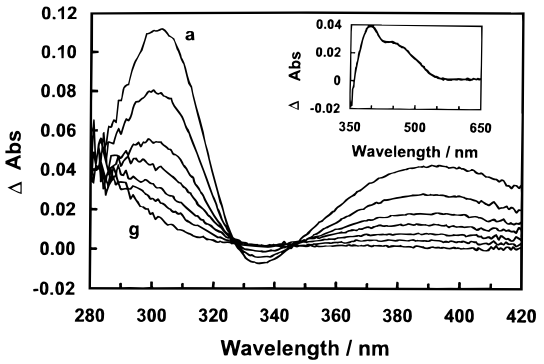


FIGURE 1: Difference spectrum of the observed transient flavin intermediate. After mixing, the anaerobic reaction mixture contained EPSP (20 μM), enzyme (30 μM), photoreduced FMN (31 μM), oxalate (1 mM), and buffer. The blank spectrum was obtained from a control experiment without substrate. Spectra (a−g) correspond to data integrated between 9−14, 19−24, 29−34, 39−44, 49−54, 59−64, and 79−84 ms, respectively. Inset: the difference spectrum at higher wavelengths when its amplitude was maximal (data integrated between 9 and 10.25 ms).

Inorganic Phosphate Analysis. Inorganic phosphate was determined in rapid acid quenched samples (100 μL per assay) in duplicate using a modified molybdate/Malachite Green method (Lanzetta et al., 1979) using Triton N101 instead of Sterox detergent. The acid quench procedure did not affect the inorganic phosphate determination. The chorismate synthase preparation used in these experiments was desalted as described above to reduce the level of adventitious inorganic phosphate.

Phosphate Dissociation. Inorganic phosphate dissociation was measured in the presence of a fluorescent phosphate binding protein (PBP; Brune et al., 1994) in real-time using stopped-flow spectrofluorimetry. All solutions contained a "phosphate mop", to remove adventitious inorganic phosphate prior to each experiment, that consisted of purine nucleoside phosphorylase (1 unit mL^{-1}) and 7-methylguanosine (1 mM).

Kinetic Simulations. Kinetics were simulated using the program KSIM 1.2 written by Neil C. Millar and available from Hi-Tech Scientific (Salisbury, U.K.).

RESULTS AND DISCUSSION

Characterization of a Flavin Intermediate. As previously reported, the difference spectrum of the transient flavin intermediate between 350 and 700 nm, relative to a control without substrate, exhibits a maximal amplitude at 395 nm after 9 ms with a shoulder at 450 nm and an isosbestic point at 359 nm (Figure 1 inset: Bornemann et al., 1995b). The basic shape of the spectrum does not change as it forms and decays.

We now report the spectral changes associated with the chorismate synthase reaction at lower wavelengths. The main panel of Figure 1 shows the difference spectra obtained during a single turnover experiment relative to a control experiment without substrate. The first spectrum shown exhibited the maximal amplitude (Figure 1a; data integrated between 9 and 14 ms). These new data show an additional isosbestic point at 327 nm and a relatively large increase in

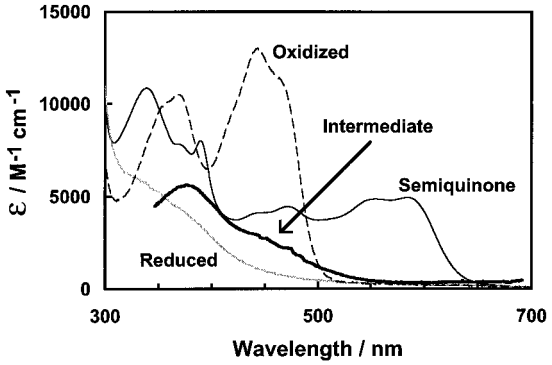


FIGURE 2: Absorbance spectra of the observed transient flavin intermediate (boldface line) and three stable redox states of chorismate synthase: oxidized (broken line); semiquinone (solid line); reduced (dotted line). The transient flavin intermediate spectrum was obtained using stopped-flow spectrophotometry when its amplitude was maximal. After mixing, the anaerobic reaction mixture contained EPSP (20 μM), enzyme (20 μM), photoreduced FMN (40 μM), oxalate (1 mM), and buffer. The transient spectrum was corrected for the excess free photoreduced FMN (20 μM). The extinction coefficient was calculated on the basis that all of the enzyme was in the form of the transient intermediate.

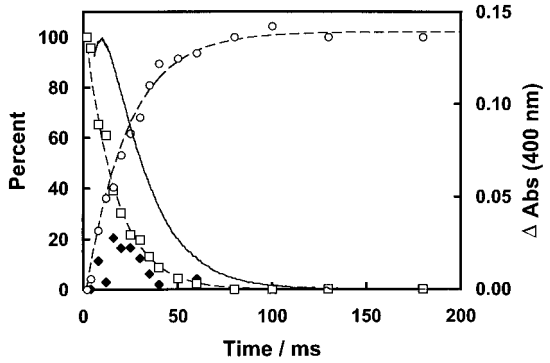


FIGURE 3: Kinetics of substrate consumption (□) and chorismate formation (○) during a single turnover experiment determined by rapid chemical quench followed by HPLC. The broken lines show best fits using single exponentials with rate constants of 60 and 45 s^{-1} for EPSP consumption and chorismate formation, respectively. A plot of the deficit in the combined yields of EPSP and chorismate (♦) indicates the possible quenching of a substrate-derived intermediate. A control experiment monitoring the formation and decay of the flavin intermediate by stopped-flow spectrophotometry under identical conditions is shown for comparison (solid line). After mixing, the anaerobic reaction mixtures contained EPSP (70 μM), enzyme (105 μM), reduced FMN (105 μM), dithionite (1 mM), and buffer.

absorbance at 310 nm. The basic shape of the spectrum above 320 nm did not change as it formed and decayed. In addition, the biphasic kinetics of the absorbance changes monitored at single wavelengths are very similar at 450, 400, and 310 nm. Therefore, there is no evidence for more than one spectrally distinct intermediate in this wavelength range. The final difference spectrum, after the single turnover event (Figure 1g: data integrated between 79 and 84 ms), shows no absorbance change above 320 nm. This result clearly shows that the flavin intermediate decays completely, within the limits of detection, and that all of the enzyme relaxes back to its resting state. At wavelengths below 320 nm, there is some residual absorbance in the last difference spectrum (Figure 1g), which remains in spectra obtained after longer times. The product of the reaction, the diene chorismate, absorbs in this region ($\lambda_{max} = 275$ nm, $\epsilon = 2630$ M^{-1} cm^{-1}) and must be responsible for this absorbance. The final difference spectrum is therefore a spectrum of chorismate. The influence of the enzyme on the absorbance spectrum of bound chorismate and the possible presence of additional intermediates that absorb in this range are unknown. It is therefore not possible to deconvolute these spectra and obtain an unambiguous difference spectrum of the flavin intermediate alone at wavelengths below 320 nm. It is also not possible to deconvolute the kinetics of chorismate formation and the formation and decay of the flavin intermediate at wavelengths below 310 nm. The observed kinetics at these low wavelengths are more complicated than those at 310 nm and above.

Figure 2 shows the actual absorbance spectrum of the transient flavin intermediate, when its concentration is maximal, in the context of the spectra of the stable oxidized, semiquinone, and reduced forms of the holoenzyme. The maximum absorbance of the flavin intermediate is at 375 nm. The extinction coefficient at this wavelength ($\epsilon = 5600$ M^{-1} cm^{-1}) is a minimum value because it was calculated assuming that all of the enzyme is in the transient form. There is significant absorbance at 520 nm that is absent in the spectrum of the oxidized holoenzyme. In addition, there is a small but significant amount of broad absorbance at longer wavelengths, extending to at least 700 nm (Figure 2).

The kinetics of the formation and decay of the flavin intermediate during a single turnover experiment with a 1.5-fold excess of enzyme over substrate are shown in Figure 3. The formation of the intermediate is so rapid at 25 °C, that about 90% of the change in absorbance is obscured by the dead time of the stopped-flow equipment (all data are corrected for the dead time of 4 ms). The best fit of the data from quadruplicate experiments using two single exponentials gives values of 160 ± 10 s^{-1} for its formation and 52 ± 2 s^{-1} for its decay (Bornemann et al., 1995b). Essentially identical rates of formation and decay are observed using EPSP and enzyme concentrations of either 70 and 105 μM (Figure 3), respectively, or 4 and 6 μM (Figure 5). Therefore, the rate of formation of the flavin intermedimate is always limited by a first-order process in the concentration range used. Moreover, the kinetics of this enzyme cannot be limited by the second-order binding step of the substrate in any of the experiments described in this study. Given the relatively small absorbance change associated with the flavin intermediate and the limit of detection of the stopped-flow instrument, reliable kinetic analyses of data obtained with substrate concentrations of less than 4 μM, where the binding of substrate may become limiting, are not possible.

Substrate Consumption and Product Formation. Figure 3 also shows the consumption of EPSP and the formation of chorismate during a single turnover experiment determined by rapid chemical quench followed by HPLC analysis. Since the chemical quench denatures the enzyme, these data must represent the sum of bound and free EPSP and the sum of bound and free chorismate. Both EPSP and chorismate are stable in the acid-quenched samples for a period of days at 4 °C, and the yield of chorismate was identical with experiments quenched after 200 ms and after 2 min. It is assumed that the acid quench does not induce any chemical reactions involving EPSP or chorsimate bound to the enzyme. A best fit using a single exponential gives a rate constant of 60 s^{-1} for the consumption of EPSP. These data fit well to a single exponential with no sign of a large burst or lag phase.

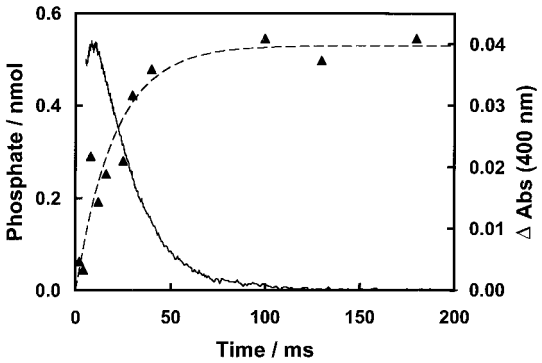


FIGURE 4: Kinetics of inorganic phosphate elimination (▲) during a single turnover experiment determined by rapid chemical quench followed by colormetric analysis in duplicate. The data are corrected for a time zero control experiment determined in quadruplicate. The broken line shows the best fit using a single exponential with a rate constant of 50 s^{-1}. A control experiment monitoring the formation and decay of the flavin intermediate by stopped-flow spectrophotometry under identical conditions is shown for comparison (solid line). After mixing, the anaerobic reaction mixtures contained EPSP (20 μM), enzyme (30 μM), reduced FMN (30 μM), dithionite (1 mM), and buffer.

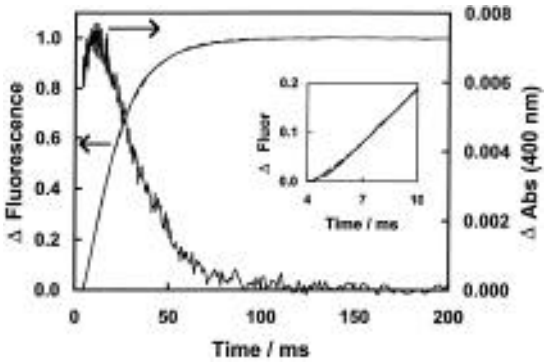


FIGURE 5: Kinetics of inorganic phosphate dissociation monitored using the PBP (smooth solid line). The smooth broken line shows an essentially coincident best fit to these data using two single exponentials of 210 ± 20 s^{-1}, describing a small lag (see inset), and 63 ± 2 s^{-1}. A control experiment monitoring the formation and decay of the flavin intermediate by stopped-flow spectrophotometry under identical conditions, except for the omission of the PBP, is shown for comparison. After mixing, the anaerobic reaction mixtures contained EPSP (4 μM), enzyme (6 μM), reduced FMN (6 μM), dithionite (500 μM), PBP (12 μM), purine nucleoside phosphorylase (1 unit mL^{-1}), 7-methylguanosine (1 mM), and buffer.

The flavin intermediate must form before EPSP is consumed because the rate of formation of the flavin intermediate (160 ± 10 s^{-1}) is clearly the more rapid of these two processes.

A best fit using a single exponential gives a rate constant of 45 s^{-1} for the formation of chorismate (Figure 3). The conversion of EPSP to chorismate may not be concerted, because the rate of chorismate formation is lower than that of EPSP consumption. The difference in the rates is not great enough to provide unequivocal proof that the reaction is not concerted. However, the EPSP and chorismate concentrations in each quenched sample were analyzed simultaneously using HPLC, ruling out any obvious systematic errors being responsible for this difference. In addition, a substrate-derived intermediate must be quenched during the rapid chemical quench experiments if the reaction were nonconcerted. The sum of the yields of EPSP and chorismate is in fact less than 100% during part of the single turnover experiment. A plot of the deficit (Figure 3) indicates the transient formation of a substrate-derived intermediate up to 20% of the active site concentration. No new compounds were identified using this HPLC separation technique. Such a quenched intermediate could coelute with any of the constituents of the reaction mixture but clearly does not coelute with either the substrate or the product. Another chromatographic separation method would be required to detect such an intermediate. In addition, it would appear that no stable flavin intermediates, such as covalent adducts, are quenched since, after oxidation in air, the flavin in all of the quenched samples exhibited normal absorbance spectra and normal elution profiles using HPLC.

Figure 4 shows the kinetics of inorganic phosphate elimination during a single turnover experiment using rapid chemical quench followed by colorimetric analysis. If inorganic phosphate were eliminated [i.e., the C(3)−O bond were cleaved] as EPSP were consumed, a best fit to the data using a single exponential should give an identical rate constant of 60 s^{-1}. The yield of inorganic phosphate from duplicate experiments is very close to that expected (0.54 nmol on the basis of EPSP). However, the phosphate analysis was close to the detection limit, and the scatter in the data leads to large errors in the fitted rate constant that is obtained (50 s^{-1}). It is therefore difficult to say whether or not inorganic phosphate elimination and EPSP consumption are synchronous. Similarly, the data are not accurate enough to conclude firmly that inorganic phosphate elimination and chorismate formation are synchronous. However, these data clearly confirm the observations of Hawkes et al. (1990) that inorganic phosphate elimination does not show a large burst or lag phase. Furthermore, the rate of elimination of phosphate is clearly slower than that for the formation of the flavin intermediate (160 ± 10 s^{-1}). This result, coupled with the observation that C(6*proR*)−H bond cleavage occurs after the formation of the intermediate (Bornemann et al., 1995b), provides strong independent evidence that the flavin intermediate forms before EPSP is consumed.

Phosphate Dissociation. Figure 5 shows the dissociation of inorganic phosphate from chorismate synthase during a single turnover experiment in real time using a coupled fluorescent assay. The assay uses a genetically engineered phosphate binding protein (PBP) that binds phosphate very rapidly (1.36×10^8 M^{-1} s^{-1}) and tightly ($K_d \sim 0.1$ μM). Binding leads to a conformational change in the protein and a 5-fold increase in the fluorescence of a covalently attached coumarin fluorophore (Brune et al., 1994). Adventitious inorganic phosphate is removed from solutions prior to each experiment by adding a "phosphate mop" consisting of 7-methylguanosine and purine nucleoside phosphorylase. It is assumed that the amount of phosphate released is stoichiometric with the amount of EPSP at the start of each experiment. Greater than 98% of the released phosphate would be bound to the PBP under these experimental conditions. The signal to noise ratio for these data is very high, and a small lag phase is clearly visible, with the initial slope being close to zero (Figure 5 inset). A similar lag was observed during multiple turnover reactions. Based on this lag, it is estimated that less than 3% of the amplitude is obscured by the dead time of the stopped-flow equipment. A best fit of the data from quadruplicate determinations using two single exponentials gives rate constants of 210 ± 20 s^{-1}, associated with the small lag, and 63 ± 2 s^{-1}, with

residuals of less than 0.5% throughout the time courses. The rate constant for the binding of phosphate to the PBP (1.36×10^8 M^{-1} s^{-1}) is sufficiently large to preclude a detectable lag between phosphate being released from chorismate synthase and binding to the PBP (12 μM) in these experiments. Despite the high signal to noise ratio associated with this technique, it is not possible to obtain reliable results at lower substrate concentrations, where substrate binding may be limiting. This is due to the limitations imposed by the binding rate constant and K_d for phosphate and the PBP.

Figure 5 also shows the kinetics of the formation and decay of the flavin intermediate under identical experimental conditions, except for the omission of the PBP. The best fit of these data gave identical values to those at the other substrate and enzyme concentrations, as described above. The presence of the "phosphate mop", and the subsequent absence of free inorganic phosphate, therefore had no effect on the kinetics of the chorismate synthase reaction. A control reaction in the presence of the PBP was not possible because the fluorophore absorbs in the same region of the spectrum as the flavin intermediate. In addition, the absorbance spectrum of the PBP changes on phosphate binding. To overcome this obstacle, a second control in the presence of wild-type PBP, that had no fluorophore attached, was performed. This gave essentially identical results to the first control described above. Therefore, the presence of the PBP had no detectable effect on the kinetics of the chorismate synthase reaction. Finally, a third control in the presence of phosphate (pH 7.5: 10 mM after mixing) also gave essentially identical results, showing that the presence of the product phosphate at this concentration had no effect on the kinetics of this single turnover experiment. This result also shows that the association rate for inorganic phosphate is very small.

Determination of k_{cat}. A value of k_{cat} of 17 s^{-1} is obtained using steady-state assays. However, the values of kinetic constants obtained using rapid reaction techniques at the protein concentrations used for the single turnover experiments are more informative and reliable since chorismate synthase specific activity is dependent on protein concentration (Hawkes et al., 1990) and the product, chorismate, is known to oxidize the holoenzyme on the minute time scale (Bornemann et al., 1995a; Macheroux et al., 1996). The single turnover experiments described above do not necessarily give any information about the overall k_{cat} of the chorismate synthase reaction. Muliple turnover experiments are required to obtain an unambiguous value.

A k_{cat} of 33 $\pm$ 3 s^{-1} was measured for the linear phase of the formation of chorismate during a multiple turnover rapid quench experiment with a 5-fold excess of EPSP over enzyme (data not shown). In similar experiments, monitoring chorismate formation using the increase in absorbance at 275 nm, a k_{cat} of 27 $\pm$ 2 s^{-1} was obtained from triplicate determinations (data not shown). The time course was complicated during the first ~10 ms, and then essentially linear during a quasi-steady-state phase for about four turnovers and finally complicated again as the substrate became exhausted. The concentration of the flavin intermediate is essentially constant throughout the quasi-steady-state phase (data not shown). The increase in absorbance at 275 nm during this phase must therefore accurately reflect the formation of chorismate. A third experiment, monitoring the dissociation of phosphate, gave a k_{cat} of 28 $\pm$ 1 s^{-1} during the linear phase of four independent experiments.

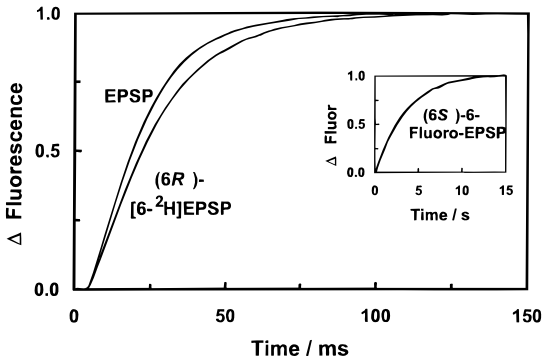


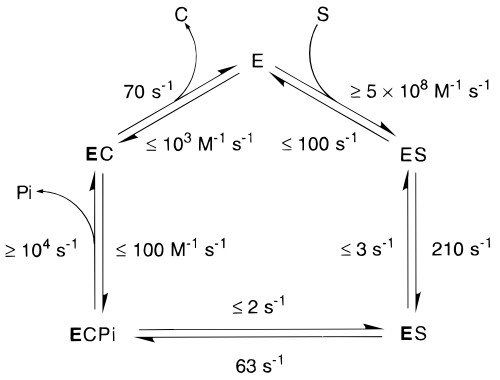
FIGURE 6: Kinetics of inorganic phosphate dissociation with (6*R*)-[6-^{2}H]EPSP monitored using the PBP. A fit of these data using two single exponentials of 200 $\pm$ 20 s^{-1}, describing a small lag, and 49 $\pm$ 2 s^{-1} is coincident with the data. The solutions were identical to those detailed in Figure 5 except (6*R*)-[6-^{2}H]EPSP was used instead of EPSP. The data obtained with EPSP (Figure 5) are shown for comparison. Inset: the kinetics of inorganic phosphate dissociation with (6*S*)-6-fluoro-EPSP monitored using the PBP. A fit of these data using one single exponential of 0.28 $\pm$ 0.01 s^{-1} is coincident with the data. The solutions were identical to those detailed in Figure 5 except (6*S*)-6-fluoro-EPSP was used instead of EPSP.

In a previous study, the elimination of inorganic phosphate was measured during a multiple turnover experiment (Hawkes et al., 1990), and a k_{cat} of 18 s^{-1} was calculated on the basis of chorismate synthase subunit concentration. However, since the flavin concentration was in fact limiting in their experiment, the true k_{cat} can be recalculated to be 27 s^{-1}. There is clearly good agreement, with all four values of k_{cat} giving a mean value of 29 $\pm$ 3 s^{-1}.

Transient Kinetics Using Substrate Analogues. An isotope effect on V_{max} of 1.13 $\pm$ 0.03 has been reported using the substrate (6*R*)-[6-^{2}H]EPSP (Bornemann et al., 1995b). No isotope effect was observed for the formation of the flavin intermediate, but an effect of 1.17 $\pm$ 0.04 was measured for its decay. The single turnover experiments above show that phosphate is released from chorismate synthase after EPSP is converted to chorismate and before the decay of the flavin intermediate. Therefore, an isotope effect is likely to be expressed on phosphate dissociation. Figure 6 shows that there is indeed an isotope effect on this process. A fit of data from five experiments using two single exponentials gives rate constants of 200 $\pm$ 20 s^{-1}, describing a small lag, and 49 $\pm$ 2 s^{-1}. It is clear that there is no discernible isotope effect on the lag phase. However, from quadruplicate determinations, the isotope effect on the second exponential was 1.27 $\pm$ 0.03. Since the (6*R*)-[6-^{2}H]EPSP was 80% deuteriated, the remainder being in the protio form, the maximum theoretical isotope effect is estimated to be 1.34 $\pm$ 0.04.

The substrate analogue (6*S*)-6-fluoro-EPSP has previously been shown to be turned over by chorismate synthase, giving 6-fluorochorismate as the product (Bornemann et al., 1995a). The V_{max} for this substrate was 2 orders of magnitude lower than that for EPSP. The rate of formation of the flavin intermediate was a little higher with this analogue (210 $\pm$ 10 s^{-1}), but its rate of decay was decreased substantially to 0.186 $\pm$ 0.004 s^{-1}. Since the fluoro substituent is likely to affect the rate of chemical steps in the catalytic mechanism, the rate of phosphate dissociation will likewise be affected.

Scheme 3



A best fit of data from five experiments using a single exponential gives a rate constant of 0.28 ± 0.01 s^{-1} with no detectable lag phase (Figure 6 inset). If there were a lag of about 210 s^{-1}, it would have a negligible effect on the progress curve since the rate constant of 0.28 ± 0.01 s^{-1} is 3 orders of magnitude smaller.

The Catalytic Cycle of Chorismate Synthase. Two possible models have been developed to illustrate the main findings of this work, to show the order of key events in the catalytic cycle and to define limits for the values of the rate constants for each model. It is not yet certain whether the chorismate synthase reaction involves a concerted or nonconcerted mechanism, so both possibilities must be considered. However, the main conclusions of this work remain the same with either mechanism. Let us first consider a model involving a concerted mechanism (Scheme 3). In this study, the holoenzyme, with the anionic form of reduced FMN bound, is formed from the apoenzyme and reduced flavin before every experiment (K_d for reduced FMN = 18 nM: Macheroux et al., 1996). The first step in the model is therefore the binding of the substrate (S) to the reduced holoenzyme of chorismate synthase (E) to form the reduced holoenzyme/substrate complex (ES). The second step must be the formation of the observed flavin intermediate (**E**S) at a rate of at least 160 s^{-1}. The formation of the flavin intermediate is a first-order process at all of the enzyme and substrate concentrations studied. Therefore, the binding of the substrate must be close to diffusion rate limitation, with an association rate constant for EPSP greater than about 5×10^8 M^{-1} s^{-1}.

The third step of this concerted model involves the consumption of EPSP, at a measured rate of 60 s^{-1}, accompanied by the formation of chorismate and elimination of phosphate (**E**CPi). Since one of the last events in the catalytic cycle is the decay of the observed flavin intermediate (see below), the flavin is still in this form. The fourth step involves the dissociation of inorganic phosphate, which must be very fast compared with the previous step because chorismate formation and phosphate dissociation are essentially concomitant. The dissociation rate constant for phosphate must therefore be greater than 10^4 s^{-1}. Since inorganic phosphate was not found to be inhibitory at a concentration of 10 mM, the association rate constant for phosphate must be less than 100 M^{-1} s^{-1}. The kinetics of phosphate dissociation are described by a rate of 63 ± 2 s^{-1} with a lag of 210 ± 20 s^{-1}. Although it is clear that EPSP consumption (60 s^{-1}) must be slower than the rate at which the flavin intermediate forms (160 ± 10 s^{-1}), the possibility

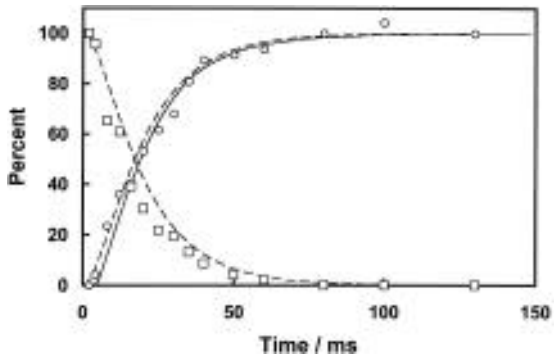


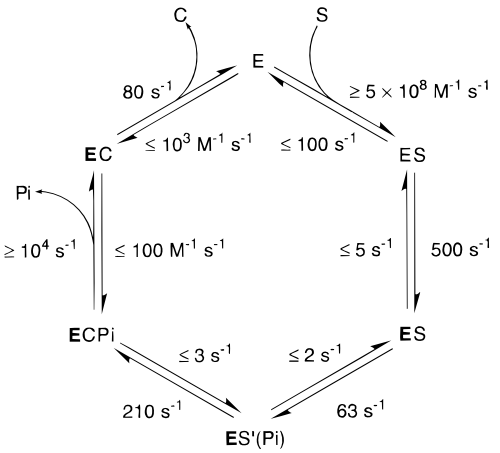
FIGURE 7: Simulation of the single turnover experiments from Figures 3 and 5 using the kinetic model for a concerted reaction from Scheme 3: EPSP consumption (□); chorismate formation (○); phosphate dissociation (solid line); simulations (broken lines). The simulated curves for phosphate dissociation and chorismate formation are coincident.

exists that a substrate-derived intermediate gives EPSP on acid quench. However, the kinetics of phosphate dissociation independently confirm the rate at which EPSP is consumed and discount this possibility. The forward rate constant for the second step must therefore be 210 ± 20 s^{-1} in this model. In addition, the dissociation rate constant for EPSP must be less than about 100 s^{-1} in order for the reaction not to be substrate-limited at the concentrations studied in the single turnover experiments. This gives a limiting value of the K_d for EPSP binding to the reduced holoenzyme of less than 0.2 μM. The relative magnitudes of the rate constants for EPSP dissociation, the second step of the model, and k_{cat} are consistent with the high commitment to catalysis observed with the *N. crassa* enzyme (Balasubramanian et al., 1991; Lauhon & Bartlett, 1994).

Since the rate at which the flavin intermediate decays is almost certainly slower (52 ± 2 s^{-1} from quadruplicate determinations) than the rate of phosphate dissociation (63 ± 2 s^{-1} from quadruplicate determinations), the decay of the observed flavin intermediate to regenerate the active resting state of the enzyme is most probably one of the last events in the catalytic cycle. Chorismate may dissociate very rapidly after it is formed and could dissociate before phosphate. However, since the flavin intermediate forms after EPSP binds to the enzyme without EPSP being consumed, it is probable that the flavin intermediate does not decay until after chorismate has been released. The decay of the flavin intermediate and chorismate dissociation have therefore been combined into one final step. The association rate constant for chorismate must be small ($\leq 10^3$ M^{-1} s^{-1}) because no product inhibition by chorismate is observed at the concentrations studied (Ramjee, 1992; Ramjee et al., 1994).

In order to show that the concerted kinetic model shown in Scheme 3 is valid, the kinetics of the chorismate synthase reaction during single and multiple turnover experiments were simulated. Figure 7 shows the results of a best fit simulation of the experimental data obtained for EPSP consumption, chorismate formation, and phosphate dissociation using the constraints discussed above. In order for the overall k_{cat} of the model to be 29 s^{-1}, the forward rate constant for the last step has to be 70 s^{-1}. This makes this final step partially rate-limiting. Studies with substrate analogues and isotope effects using the *N. crassa* enzyme support such a final partially rate-limiting protein confor-

Scheme 4



mational change and/or chorismate dissociation step (Lauhon & Bartlett, 1994).

There is no evidence of overall reversibility in the chorismate synthase reaction (Balasubramanian et al., 1995). The only step that is known to be reversible is the binding of EPSP. The kinetic model described here therefore represents the simplest concerted model that can account for the experimental observations. Since it is not known which steps in the kinetic model are irreversible, the maximum values of rate constants for the reverse reactions which do not lead to a detectable change in the simulations, with unaltered forward rate constants, were also estimated (Scheme 3). Although the simulations appear to be reasonable using this concerted model (Figure 7), they suffer from the introduction of too great a lag for EPSP consumption and too small a lag for chorismate formation and phosphate dissociation. This limitation is dependent on the model and not on the values of the rate constants used. In addition, the data for the formation and decay of the flavin intermediate cannot be simulated using this model, whichever kinetic species are assumed to contribute to the spectral changes.

An alternative kinetic model involving a nonconcerted mechanism is shown in Scheme 4. This model is very similar to the concerted model, except that it has an extra chemical step, that may involve phosphate elimination, and one additional intermediate involving an enzyme-bound substrate-derived intermediate [**E**S′(Pi)]. The new chemical step must occur at 210 s^{-1} for the appropriate lag to be absent in EPSP consumption and to be present in chorismate formation and phosphate dissociation. The best fit simulation using the nonconcerted model gives curves in good agreement with the experimental data (Figure 8). In order to obtain a satisfactory fit, the rate at which the flavin intermediate forms must be about 500 s^{-1}. In addition, the last step describing chorismate dissociation and the decay of the flavin intermediate must occur at a rate of about 80 s^{-1} for the cycle to be consistent with the experimental value of k_{cat}. The simulated curves for EPSP consumption, chorismate formation, and phosphate dissociation give particularly good fits using this nonconcerted model. In addition, the simulated curve for the putative substrate-derived intermediate gives a reasonable fit. More importantly, the simulated curve for the flavin intermediate fits well to the experimental data, assuming that all of the kinetic species that are in the form of the flavin intermediate [**E**S, **E**S′(Pi), **E**CPi, and **E**C] have identical spectroscopic properties. This assumption is reasonable since evidence for only one flavin intermediate has been obtained. The nonconcerted model fits the observed data better than the concerted model, but this alone does not allow a conclusive distinction to be made between them. Although the absolute values of all of the rate constants are not yet defined, their limiting values determined in this work do allow reasonable simulations of the data and valuable predictions to be made for future experiments.

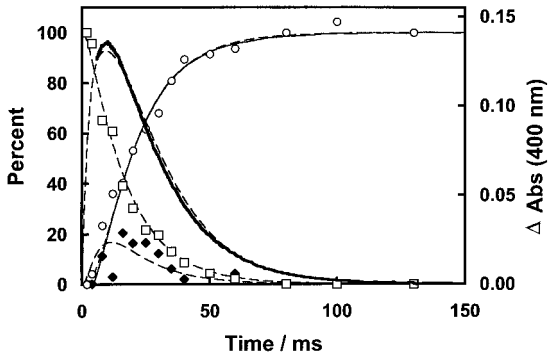


FIGURE 8: Simulation of the single turnover experiments from Figures 3 and 5 using the nonconcerted kinetic model from Scheme 4: flavin intermediate (boldface solid line); EPSP consumption (□); a possible substrate-derived intermediate (♦); chorismate formation (○); phosphate dissociation (narrow solid line); simulations (broken lines). The simulated curves for phosphate dissociation and chorismate formation are coincident with each other and the observed curve for phosphate dissociation.

Confidence in the kinetic models is gained by considering the observations with (6*R*)-[6-^{2}H]EPSP. The effect on the kinetics of phosphate dissociation shows that the forward rate constant for EPSP consumption is decreased from 63 to 49 s^{-1}. If the kinetic models include this decreased rate constant, the simulated curves for the flavin intermediate give best fits using two single exponentials with unchanged formation rates and decay rates with isotope effects of about 1.18. This matches the experimental observation that the formation rate was unchanged and the decay rate exhibited an isotope effect of 1.17 ± 0.04 (Bornemann et al., 1995b). Similarly, the simulated kinetic isotope effect of 1.13 on k_{cat} with either model is identical to the observed isotope effect of 1.13 ± 0.03. Although the measured isotope effects on the decay of the flavin intermediate and k_{cat} are indistinguishable when considered in light of experimental errors, the apparent differences in these isotope effects may be real. This result does not help to distinguish between the models, but it does help to validate them.

This isotope effect using (6*R*)-[6-^{2}H]EPSP could be a small primary effect on C(6*proR*)−H cleavage, which could be expressed whether or not the reaction were concerted. Alternatively, if phosphate elimination is the first chemical step in a nonconcerted reaction, this isotope effect could be a large secondary vinylogous β-effect on C(3)−O bond cleavage, as described by Bornemann et al. (1995b). The observation of an analogous secondary β-isotope effect on V_{max} using [4-^{2}H]EPSP would provide very strong evidence for a nonconcerted reaction, where C(3)−O cleavage precedes C(6*proR*)−H cleavage. If such an isotope effect were expressed on the decay of the flavin intermediate and the main phase of phosphate dissociation, as has been observed with (6*R*)-[6-^{2}H]EPSP, C(3)−O cleavage can only be assigned to the third step of the nonconcerted kinetic model.

Nature of the Flavin Intemediate. The experiments described in this paper show that the flavin intermediate is not simply associated with the conversion of substrate to product. It must form before any chemical steps involving EPSP have occurred, most probably decays on chorismate dissociation, and must be associated with several kinetic species of the catalytic cycle. It can therefore not be a C(4a)-flavin−EPSP adduct or a charge transfer complex between the flavin and a substrate-derived intermediate. The reduction of a disulfide to form a thiolate nucleophile is not possible since disulfides are not required for activity (Ramjee et al., 1991; Bornemann et al., 1996). Therefore, the flavin intermediate cannot be a C(4a)-flavin−cysteine adduct either. The spectral changes must be associated primarily with events such as protonation of the reduced flavin, a conformational change in the protein, or a charge transfer complex between reduced flavin and an aromatic amino acid. This does not rule out the direct role of flavin in catalysis. It has been suggested that binding of the substrate to the reduced holoenzyme is accompanied by protonation of the flavin (Macheroux et al., 1996). This provides a role for flavin in catalysis since protonation could lower its redox potential and promote radical chemistry such as that shown in Scheme 2a. The spectral changes associated with the flavin intermediate are qualitatively similar to those seen on flavin protonation (Ghisla et al., 1974), and interestingly, a study of the effect of pH on reduced *Desulfovibrio vulgaris* flavodoxin shows quantitatively similar spectral changes (Yalloway et al., 1995). It therefore seems increasingly likely that the spectral changes are associated with protonation of the reduced flavin.

If the reaction were concerted, the role of the flavin would be obscure. The reaction is more likely to be nonconcerted because the studies with 5-deaza-FMN strongly suggest that the flavin is directly involved in the catalytic mechanism (Lauhon & Bartlett, 1994; Bornemann et al., 1995c). The effect of using (6*S*)-6-fluoro-EPSP on the kinetics of phosphate dissociation confirms other studies with this substrate analogue that suggest the nonconcerted mechanisms shown in Scheme 2 are the most likely (Bornemann et al., 1995a). It is possible that the electron-rich fully reduced flavin contributes to the stabilization of the cationic intermediate in the mechanism shown in Scheme 2b. An alternative involves the formation of a flavin semiquinone in the radical mechanism shown in Scheme 2a. A neutral flavin semiquinone radical is not detectable in the single turnover experiments with EPSP. Anionic flavin semiquinone radicals, on the other hand, typically have a major peak at 375 nm ($\epsilon = 20\,000\ M^{-1}\ cm^{-1}$), a smaller peak at 480 nm, a weak flat band at longer wavelengths, and an isosbestic point with fully reduced flavin at 340 nm (Massey & Palmer, 1966). Although there is spectral evidence for only one flavin intermediate, these characteristics are sufficiently similar to those of the observed flavin intermediate to allow the possibility of such a species contributing, in part, to the observed spectral changes.

Overall Conclusions. This paper describes observations that are consistent with a nonconcerted chorismate synthase reaction. We favor the nonconcerted model because: it takes into account the transient deficit in the combined yields of unreacted substrate and product as seen using rapid chemical quench followed by simultaneous HPLC analysis; it can simulate the formation and decay of the flavin intermediate; and it can simulate the lag observed for phosphate dissociation without introducing a lag in EPSP consumption. Although these studies do not conclusively distinguish between the proposed nonconcerted mechanisms, they are consistent with the two that are most likely (Scheme 2). The isolation and characterization of a substrate-derived intermediate will help define the reaction mechanism. In addition, the observation of an isotope effect using [4-^{2}H]-EPSP would provide very strong evidence for a nonconcerted reaction, where C(3)−O cleavage must precede C(6*proR*)−H cleavage. What is clear from this work is that the flavin intermediate is not simply associated with the conversion of substrate to produce because it forms before the substrate is consumed. The transient spectral changes must be associated primarily with events such as protonation of the reduced flavin, a charge transfer complex between reduced flavin and an aromatic amino acid, or a conformational change in the protein. This does not rule out the direct role of flavin in catalysis.

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