Escherichia coli Chorismate Synthase Catalyzes the Conversion of (6S)-6-Fluoro-5-enolpyruvylshikimate-3-phosphate to 6-Fluorochorismate

IMPLICATIONS FOR THE ENZYME MECHANISM AND THE ANTIMICROBIAL ACTION OF (6S)-6-FLUOROSHIKIMATE*

(Received for publication, April 17, 1995, and in revised form, June 30, 1995)

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Chorismate synthase catalyzes the conversion of 5-enolpyruvylshikimate-3-phosphate to chorismate. It is the seventh enzyme of the shikimate pathway, which is responsible for the biosynthesis of aromatic metabolites from glucose. The chorismate synthase reaction involves a 1,4-elimination with unusual anti-stereochimistry and requires a reduced flavin cofactor. The substrate analogue (6S)-6-fluoro-5-enolpyruvylshikimate-3-phosphate is a competitive inhibitor of Neurospora crassa chorismate synthase (Balasubramanian, S., Davies, G. M., Coggins, J. R., and Abell, C. (1991) J. Am. Chem. Soc. 113, 8945–8946). We have shown that this analogue is converted to 6-fluorochorismate by Escherichia coli chorismate synthase at a rate 2 orders of magnitude slower than the normal substrate. The decreased rate of reaction is consistent with the destabilization of an allylic cationic intermediate. The formation of chorismate and 6-fluorochorismate involves a common protein-bound flavin intermediate although the fluoro substituent does influence the spectral characteristics of this intermediate. The fluoro substituent also decreased the rate of decay of the flavin intermediate by 280 times. These results are consistent with the antimicrobial activity of (6S)-6-fluoroshikimate not being mediated by the inhibition of chorismate synthase but by the inhibition of 4-aminobenzoic acid synthase as previously proposed (Davies, G. M., Barrett-Bee, K. J., Jude, D. A., Lehan, M., Nichols, W. W., Pinder, P. E., Thain, J. L., Watkins, W. J., and Wilson, R. G. (1994) Antimicrobial Agents and Chemotherapy 38, 403–406).

Chorismate synthase (EC 4.6.1.4) is the seventh enzyme of the shikimate pathway (1, 2) and catalyzes the conversion of 5-enolpyruvylshikimate-3-phosphate to chorismate in the seventh enzyme of the shikimate pathway, which is responsible for the biosynthesis of aromatic metabolites such as aromatic amino acids and folate cofactors. The chorismate synthase reaction involves the 1,4-elimination of phosphate and the C-(6proR) hydrogen with overall anti-stereochimistry (3–5). A concerted elimination appears to be unlikely since studies with model systems (6, 7) and molecular orbital considerations (8, 9) suggest that concerted 1,4-eliminations with syn-stereochimistry are favored. For these reasons, several non-concerted mechanisms have been suggested for the chorismate synthase reaction (10). Proposed mechanisms include an X-group mechanism, involving the nucleophilic attack of the C-1 position of the substrate (5) (Scheme 2d), and a cationic mechanism, involving the step-wise loss of phosphate and a proton (11, 12) (Scheme 2a). An allylic rearrangement of phosphate followed by a 1,2-elimination (13) has been discounted because the intermediate iso-EPSP is a competitive inhibitor rather than a substrate of the Neurospora crassa enzyme (14).

Reduced FMN is required for enzyme activity despite there being no overall reduction or oxidation in the conversion of EPSP to chorismate (15–19). The role of flavin is not yet clear but it appears to be directly involved in catalysis. A transient flavin intermediate has been characterized by uv/visible spectroscopy during single and multiple turnover experiments using the enzyme from Escherichia coli (20, 21). It has been suggested that this spectrum is consistent with a charge transfer complex or a C-4a-flavin adduct (20). However, such a perturbation of the reduced flavin spectrum could, at least in part, be due to noncovalent interactions between the substrate and the flavin. The absence of detectable activity of the N. crassa (22) and E. coli (23) enzymes reconstituted with reduced 5-deaza-FMN provides additional evidence that reduced flavin is chemically, and not just structurally, involved in turnover. In the presence of the inhibitor (6R)-6-fluoro-EPSP the E. coli enzyme forms a protein-bound flavin semiquinone, suggesting the possibility of radical intermediates during normal turnover (24). A radical mechanism involving the initial abstraction of a hydrogen atom from the C-6 position of the substrate (11, 22, 25) seems unlikely since there are no obvious candidates for a catalytic center that can accept single electrons, such as transition metal ions (24), particularly given the requirement for fully reduced flavin for activity.

*This work was supported by ZENECA Pharmaceuticals, ZENECA Agrochemicals, and the Biotechnology and Biological Sciences Research Council/Medical Research Council/Department of Trade and Industry, Link Programme in Protein Engineering (to S. B.) and Science and Engineering Research Council and ZENECA Agrochemicals CASE studentships (to M. K. R. and S. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: EPSP, 5-enolpyruvylshikimate-3-phosphate; HPLC, high performance liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid.
The enzymes of the shikimate pathway are potential targets for antibiotics and herbicides because they are present in bacteria, fungi, and plants but not in mammals. Chorismate synthase is a prime candidate because it has been identified as one of the rate-limiting enzymes of this pathway (26). The 6R- and 6S-isomers of 6-fluoroshikimate are antibacterial agents displaying minimum inhibitory concentrations of 64 and 0.5 μg ml⁻¹ against E. coli K12, respectively (27). Both isomers of 6-fluoroshikimate are converted in vitro to 6-fluoro-EPSP by E. coli shikimate kinase and EPSP synthase (28). Since (6R)-6-fluoro-EPSP lacks the (6one-electron oxidation of the rate-limiting enzymes of this pathway (26). The 6S-isomers of 6-fluoroshikimate-3-phosphate (12.4), (6S)-6-fluoro-chorismate (17.8), and 6-fluorochorismate (20.6) were eluted at a flow rate of 1.5 ml min⁻¹.

Spectrophotometric Assays—The formation of the product diene chorismate (25) (30.6 cm⁻¹) was monitored as described previously (33) and substrate concentrations were determined using this method. Aerobic assay mixtures contained substrate (50 μM), FMN (10 μM), potassium oxalate (1 mM), and buffer. The flavin was photoreduced before the addition of enzyme giving a final volume of 500 μl.

Phosphate Assays—Inorganic phosphate was determined using a modified molybdate-malachite green method (34) using Triton N101 instead of Sterox detergent.

RESULTS

Fig. 1 shows the HPLC of an anaerobic reaction mixture containing (6S)-6-fluoro-EPSP and reduced flavin before and after the addition of E. coli chorismate synthase. The fluorinated substrate analogue was converted to a single product that had a retention time (20.6 min) different from that of chorismate (25.0 min). A stoichiometric liberation of inorganic phosphate and an increase in absorbance at 275 nm accompanied this reaction. The concentration of the substrate analogue was estimated using the assumption that the product had the same extinction coefficient at 275 nm as the diene chorismate (2630 m⁻¹ cm⁻¹). On the basis of this estimation, the yield of free phosphate was 96.1%.

MATERIALS AND METHODS

All chemicals and biochemicals were of the highest grade available and unless otherwise stated were purchased from Sigma (Poole, Dorset, United Kingdom). Sodium dithionite was purchased from B. D. H. Chemicals (Poole, Dorset, U.K.). All spectrophotometric measurements were obtained with a 1-cm path length.

EPSP and (6S)-6-Fluoro-EPSP—The potassium salt of EPSP was prepared essentially as described previously (30). (6S)-6-Fluoroshikimic acid (31) was provided by ZENECA Pharmaceuticals and was converted to (6S)-6-fluoro-EPSP using shikimate kinase and EPSP synthase as described previously (28) with the following modifications. The reactions were monitored by HPLC and the product was purified using HPLC followed by isolation as the dibarium salt without the need for degradation of the ATP and ADP using apyrase.

Experimental Conditions—All experiments were performed at 25 °C and solutions were buffered using 50 mM MOPS/KOH, pH 7.5, unless otherwise stated.

Enzyme Purification—Recombinant E. coli chorismate synthase (subunit Mr = 39000 (17, 18)) was purified as reported previously (32) to a specific activity of 32 μmol of chorismate produced min⁻¹ mg⁻¹.

HPLC—Compounds were separated using a Phenomenex U.K. Ltd. Selectosil 5 SAX semi-preparative column (Macclesfield, SK10 2BN, U.K.: 250 × 10 mm) fitted with a guard column (50 × 10 mm) with an isocratic eluant of 1 mM ammonium acetate, pH 6.2. EPSP (retention time 16.0 min), chorismate (25.0), (6S)-6-fluoroshikimate (11.6), (6S)-6-fluorochorismate-3-phosphate (12.4), (6S)-6-fluoro-chorismate (17.8), and 6-fluorochorismate (20.6) were eluted at a flow rate of 1.5 ml min⁻¹.

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Phosphate Assays—Inorganic phosphate was determined using a modified molybdate-malachite green method (34) using Triton N101 instead of Sterox detergent.

Stopped-flow Spectrophotometry—Single turnover experiments were carried out using a Hi-Tech Scientific SF-51 stopped-flow spectrophotometer installed and operated in an aerobic glove box as described previously (35). Aerobic substrate solution was mixed with anaerobic enzyme solution containing FADH₂ 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 coupled to the SF-51 stopped-flow sample handling unit. Kinetic data were fitted using the Hi-Tech Scientific LS-2 v2.3b6 software.

NMR Spectroscopy—NMR spectra were obtained using a JEOL 270 MHz GXS. The resonances for the 31P, 33P, and 31F resonances were HOD (4.6), P(OMe)₃, and CFCl₃, respectively. Coupling constants (J) are in Hz. (6S)-6-fluoro-EPSP, δ₁ (270.05 MHz, 50 mM potassium phosphate buffer, pH 7.0, Lit. (28) 6.46 (1 H, d, J = 3.5, 2-CH), 5.19 (1 H, dd, J = 4.9, 4.6, J₁₀₋₁₉ = 47.1, (6R)-CH) 5.06 (1 H, broad s, (proE)-CH), 3.84 (1 H, dd, J = 3.3, 3.4, J₄₋₅ = 8.3, 4-CH), and the 3-CH, 5-CH and (proE)-CH signals were obscured by the HOD peak. δ₁ (254.05 MHz) 147.35 (1F, dd, J₁₀₋₁₉ = 48.2, J₁₊₂ = 13.4, 6-CH), δ₁ = 135.8, 6-fluorochorismic acid, 4, δ₁ (50 mM MOPS buffer, pH 7.5) 6.09 (1 H, d, J = 2.3, 9.8, J₉₋₁₀ = 7.2, 2-CH), 5.70 (1 H, d, J = 3.4, 4.6, J = 2.2, 9.7, 3-CH), 5.14 (1 H, d, J = 3.2, 2.9, (proE)-8-CH), 4.72 (1 H, dd, J = 4.5, 3.9, J₉₋₁₀ = 7.3, measured values are tentative due to overlap of HOD peak, 5-CH), 4.34 (1 H, broad dd, J = 3.4, 4.6, J = 4.5, 7.4, J₉₋₁₀ = 6.8, 4-CH), and the (proE)-8-CH signal was obscured by the HOD peak. δ₁ = 111.39 (dd, J₁₀₋₁₉ = 310.0, 81.3, 6-CH).
the signals associated with the starting material and the formation of new signals consistent with 6-fluorochorismate. The 2-CH, 3-CH, 4-CH, and (proE)-8-CH 1H signals of the product were well resolved (Fig. 2). The HOD peak obscured the 5-CH resonance partially and the (proZ)-8-CH resonance completely. The product clearly retained the enolpyruvyl side chain as the (proE)-8-CH signal remained essentially unchanged. Homonuclear 1H decoupling experiments confirmed the connectivities between the resonances associated with positions 2-CH through to 5-CH. The double doublet 19F resonance of (6S)-6-fluoro-EPSP at −174.35 ppm disappeared (Fig. 3, inset) giving rise to a new quartet at −111.39 ppm (Fig. 3). Finally, the 31P singlet resonance of (6S)-6-fluoro-EPSP at −135.8 ppm was replaced by one associated with inorganic phosphate at −136.9 ppm (data not shown).

The above data clearly establish that (6S)-6-fluoro-EPSP is an alternative substrate of E. coli chorismate synthase that yields 6-fluorochorismate as the only detectable product. (6S)-6-Fluoro-EPSP is therefore not just a competitive inhibitor of this enzyme, as has been reported for the N. crassa chorismate synthase (28).

A time course monitoring the formation of 6-fluorochorismate by an increase in absorbance at 275 nm showed that the initial rate of reaction was 270 ± 20 times lower than that observed with the normal substrate (data not shown). There was curvature in the time course such that toward the end of the reaction the rate slowed to 370 ± 30 times lower with respect to EPSP. Finally, the reaction stopped abruptly as the substrate analogue became exhausted. The sensitivity of the assay did not allow the determination of the K_m, suggesting its value was at least 5-fold lower than that for EPSP (1.3 μM (33)).

When the enzyme was incubated with reduced flavin and either 6-fluorochorismate or chorismate, the slow oxidation of a proportion of the enzyme-bound flavin to the semiquinone was observed over a period of minutes. This reaction may be responsible for the curvature in the time course of the conversion of (6S)-6-fluoro-EPSP. A detailed investigation of this curious oxidative reaction, which is similar to that seen with (6R)-6-fluoro-EPSP (24), is beyond the scope of the present paper. It is clear, however, that the oxidative reaction is slower than turnover with (6S)-6-fluoro-EPSP and that these processes are independent.

Single turnover experiments with a 1.5-fold molar excess of enzyme over (6S)-6-fluoro-EPSP gave spectroscopic changes associated with the formation and decay of a flavin intermediate (Fig. 4). The difference spectrum of the intermediate was similar but not identical to that observed using EPSP (Fig. 5). The Δmax was somewhat decreased and shifted from 395 to 385 nm. The shoulder at 445 nm became a more discrete peak at 450 nm and the isosbestic point at 355 nm was shifted slightly to 352 nm. The fluoro substituent clearly has some influence on the spectral characteristics of the intermediate. With either substrate the spectrum changed only in intensity and not in shape throughout the course of the reaction.

Fitting the kinetic data obtained with (6S)-6-fluoro-EPSP using two single exponentials gave values of 210 ± 10 and 0.186 ± 0.004 s⁻¹ for the formation and decay of the intermediate, respectively (Fig. 4). Values of 160 and 52 s⁻¹, respectively, have been obtained using data with the normal substrate, EPSP (see Fig. 2 in Ref. 32). The formation of the intermediate is therefore 3.3-fold more rapid with the substrate analogue under these conditions. Such a rapid rise in absorbance results in much of the change occurring within the 4-ms dead time of the stopped-flow spectrophotometer. The rate of decay of the intermediate was 280 ± 10 times slower than that observed for EPSP.

**DISCUSSION**

We have shown that (6S)-6-fluoro-EPSP is converted to 6-fluorochorismate by E. coli chorismate synthase at a rate between 270 and 370 times slower than EPSP. This is in contrast to the observation that this analogue is a competitive inhibitor of the N. crassa enzyme (28). If it is a substrate of the N. crassa enzyme it is turned over at least 500 times slower than EPSP (28). This is one of several differences between the enzymes from these sources. For example, carbon-(6proR)-hydrogen bond breaking does not contribute significantly to rate limitation with the E. coli enzyme (6proRδV = 1.13 ± 0.03 (32)) but is partially rate-limiting with the N. crassa enzyme (6proRδV = 2.7 ± 0.2 (29), 2.64 ± 0.02 (22)). The most striking difference is that the E. coli enzyme is monofunctional (17) while the N. crassa enzyme is bifunctional, having an additional NADPH-dependent flavin reductase activity (16, 17).

The formation of chorismate and 6-fluorochorismate appear to share a common flavin intermediate although the fluoro substituent does influence its spectral characteristics. No other intermediates, such as a flavin semiquinone radical, were detected during the single turnover experiment. With EPSP, the rate-limiting step occurs after the formation of the flavin intermediate and could be phosphate cleavage (12), the decay of the flavin intermediate, or the release of either product. Carbon-(6proR)-hydrogen bond breaking occurs after the formation of the flavin intermediate but this is not the rate-limiting step with EPSP (32). Although the rate-limiting step is not necessarily the same with the fluorinated substrate analogue, it clearly still occurs after the formation of the flavin intermediate.

The decreased rate of decay of the flavin intermediate with the fluorinated substrate did not result in an obvious increase in its transient concentration. This may indicate that essentially all of the enzyme-bound FMN is in the form of the flavin intermediate during a single turnover with either substrate. However, it is difficult to compare precisely the transient concentrations because their difference spectra are not identical. It appears that the initial rate of turnover during the continuous assay and the rate of decay of the intermediate were decreased by similar amounts. It is therefore likely that steps after the decay of the flavin intermediate, which may include product release, are not significantly rate-limiting with either substrate. A similar conclusion was drawn from deuterium kinetic isotope effect studies (32).

The decreased rate of reaction is consistent with the electron withdrawing fluoro substituent destabilizing an allylic cationic intermediate (12) that would be generated by the loss of phosphate from the substrate (Scheme 2a). In a comparable system, substrate analogues were used to establish the mechanism of farnesylpyrophosphate synthetase (36). A 57-fold decrease in
the rate of turnover using a monofluorinated analogue was observed. The resultswere interpreted as strong evidence for a stepwise, rather than a concerted condensation reaction, involving an allylic cationic intermediate with the fluoro substituent adjacent to the allylic system. An allylic cationic intermediate in the chorismate synthase reaction would also be destabilized, in part, by the negative hyperconjugative effect of the adjacent fluoro substituent (37). It is not clear what the nature of the observed flavin intermediate is with such a cationic mechanism, but the electron-rich reduced FMN could have an important role as part of an active site that is capable of stabilizing a cation. The formation and decay of a cationic intermediate would not have to be concomitant with the formation and decay of the observed flavin intermediate. Therefore, interactions between a cationic intermediate and the flavin may not be solely responsible for the transient changes in the flavin spectrum.

The negative hyperconjugative effect of the fluorine could destabilize an allylic radical intermediate that would be formed by a mechanism involving an additional one-electron reduction (Scheme 2b). The overall influence of fluorine on the stability of an adjacent radical is difficult to predict, but such a radical mechanism would provide a role for the fully reduced flavin cofactor. It is possible that a flavin semiquinone does not accumulate to an observable level during the single turnover experiments. The nature of the observed flavin intermediate therefore remains obscure with this mechanism also.

Assuming the withdrawing effect of the 6-fluoro substituent outweighs its donating effect, the reaction is unlikely to involve the initial deprotonation of the C-6 position to form an allylic anionic intermediate (Scheme 2c) because the fluorine would not be expected to decrease the rate of reaction. There is no obvious requirement for flavin in this mechanism. The alternative X-group mechanism (5) (Scheme 2d) also appears unlikely since the electron withdrawing fluorine substituent would facilitate the rate-limiting (12) nucleophilic attack of the substrate at C-1. In addition, reduced FMN is unlikely to be the X-group, because the resultant flavin adduct (flavin C-4a or N-5 adduct, for example) would be severely sterically disfavored. The reduction of a disulfide by the flavin to form a thiolate nucleophile has also been discounted (20).

(6S)-6-Fluoroshikimate is converted to 6-fluoro-EPSP by E. coli shikimate kinase and EPSP synthase (28) and we have shown 6-fluorochorismate to be produced in the presence of E. coli chorismate synthase. The reactions provide a convenient method for the preparation of the new compound 6-fluorochorismate. In a previous study, the susceptibility of E. coli to the
antimicrobial agent (6S)-6-fluoroshikimate was overcome by
the addition of 4-aminobenzoic acid and not by aromatic amino
acids (27). It is therefore clear that the antimicrobial activity of
(6S)-6-fluoroshikimate is not mediated by the inhibition of cho-
rismate synthase by (6S)-6-fluoro-EPSP. 6-Fluorochorismate
most probably inhibits 4-aminobenzoic acid synthase as pro-
duced by Davies et al. (27). The PabA and PabB proteins of
4-aminobenzoic acid synthase are responsible for the conver-
sion of chorismate to the 4-aminobenzoate precursor 4-ami
no-4-deoxychorismate (38). The mechanism of this reaction may
involves the nucelophilic attack by an enzyme active site residue
at the C-6 position of chorismate, resulting in the loss of the
4-hydroxyl group (1). This would be followed by amination at
the C-4 position, with overall retention of configuration at C-4,
and regeneration of the active site nucleophile. It is possible
that the reaction with 6-fluorochorismate would result in the
loss of hydrogen fluoride before the amination step, leading to
an aromatic dead-end complex. Alternatively, a fluoride ion
may be lost upon amination rather than the regeneration of
the enzyme nucleophile. The covalent modification and irreversible
inactivation of enzymes using fluorinated substrates are well
known (39).

Acknowledgments—We are grateful to Drs. Gareth M. Davies, Tim R.
Hawkes, Terry Lewes, and Wright W. Nichols for helpful discussions
and to Dr. S. A. Fairhurst, C. J. Macdonald, and B. Wright for NMR
spectroscopic analysis.

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J. Biol. Chem. 1995, 270:22811-22815.
doi: 10.1074/jbc.270.39.22811

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