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A Secondary β Deuterium Kinetic Isotope Effect in the Chorismate Synthase Reaction

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Chorismate synthase (EC 4.6.1.4) is the shikimate pathway enzyme that catalyzes the conversion of 5-enolpyruvylshikimate 3-phosphate (EPSP) to chorismate. The enzyme reaction is unusual because it involves a *trans*-1,4 elimination of the C-3 phosphate and the C-6 *proR* hydrogen and it has an absolute requirement for reduced flavin. Several mechanisms have been proposed to account for the cofactor requirement and stereochemistry of the reaction, including a radical mechanism. This paper describes the synthesis of [4-^{2}H]EPSP and the observation of kinetic isotope effects using this substrate with both *Neurospora crassa* and *Escherichia coli* chorismate synthases. The magnitude of the effects were $^D(V) = 1.08 \pm 0.01$ for the *N. crassa* enzyme and 1.10 ± 0.02 on phosphate release under single-turnover conditions for the *E. coli* enzyme. The effects are best rationalised as substantial secondary β isotope effects. It is most likely that the C(3)–O bond is cleaved first in a nonconcerted E1 or radical reaction mechanism. Although this study alone cannot rule out a concerted E2-type mechanism, the C(3)–O bond would have to be substantially more broken than the *proR* C(6)–H bond in a transition state of such a mechanism. Importantly, although the *E. coli* and *N. crassa* enzymes have different rate limiting steps, their catalytic mechanisms are most likely to be chemically identical.

Key Words: chorismate synthase; kinetic isotope effect.

INTRODUCTION

Chorismate synthase (EC 4.6.1.4) is the shikimate pathway enzyme that catalyzes the conversion of 5-enolpyruvylshikimate 3-phosphate (EPSP) to chorismate (*1*) (Scheme 1). It is a potentially attractive target for herbicides and antimicrobials because it is present in bacteria, fungi, and plants, but not in mammals. The enzyme is unusual because it has an absolute requirement for reduced flavin (*2–4*). In addition,

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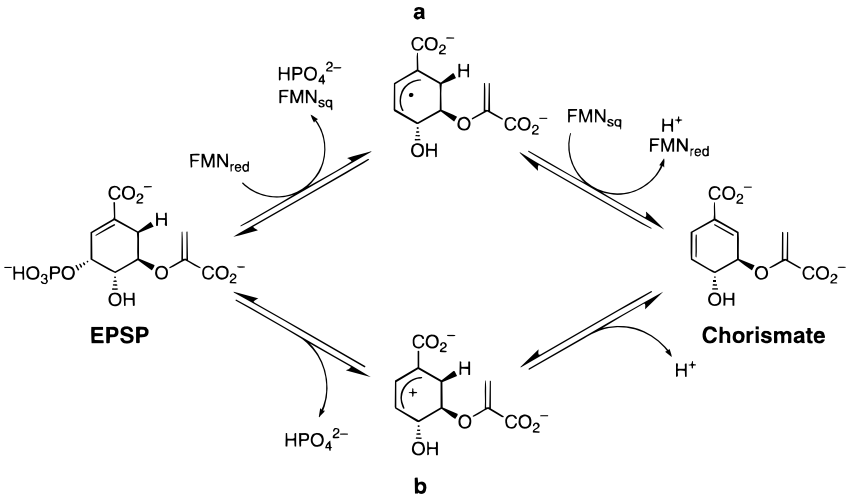


SCHEME 1

the reaction involves an unusual *trans*-1,4 elimination of the C-3 phosphate and the C-6 *proR* hydrogen (*5*–*7*), indicating that the reaction may be nonconcerted. Several mechanisms have been proposed over a number of years to account for the cofactor requirement and stereochemistry of the reaction. These have been extensively reviewed (*1, 8, 9*).

The strongest evidence for a nonconcerted reaction comes from kinetic isotope effect studies (*10*) and transient kinetics with the natural substrate (*11*). In addition, studies using the slow substrate analogue (6*S*)-6-fluoro-EPSP (*12*) indicate a nonconcerted mechanism involving either a radical or cationic intermediate (Schemes 1a and 1b, respectively). Furthermore, the formation of a flavin semiquinone radical with the substrate analogue (6*R*)-6-fluoro-EPSP (*13*) leading to phosphate cleavage (*14*) and the lack of activity with 5-deaza-FMN (*15, 16*) strongly support the radical mechanism. All of this evidence taken together makes the radical mechanism the most attractive explanation for both unusual aspects of this enzyme. Objections to the radical mechanism could be raised given that other studies with model chemical systems have shown that C(6)–H bond-cleavage could precede C(3)–O cleavage (*17*). Moreover, electrochemical studies have indicated that the reduction of model compounds in solution leads to decarboxylation rather than phosphate elimination (*18*).

A small but significant kinetic isotope effect (1.13 ± 0.03) has previously been observed using (6*R*)-[6-^{2}H]EPSP with the *Escherichia coli* enzyme (*19*). This effect can be most easily interpreted as a small primary effect expressed on *R* C(6)–^{2}H bond-cleavage. However, with this substrate, it is not possible to distinguish between this interpretation and an alternative, involving a secondary vinylogous β effect expressed on C(3)–O cleavage. This paper describes the synthesis of [4-^{2}H]EPSP and the observation of a kinetic isotope effect using this substrate with both *Neurospora crassa* and *E. coli* chorismate synthases. This effect is best rationalized as a secondary β isotope effect, providing new evidence for the order of bond-cleavage.

EXPERIMENTAL

Materials and Methods

All chemicals and biochemicals were of the highest grade available and unless otherwise stated were purchased from Sigma Chemical Company (Poole, Dorset, UK). Sodium dithionite was purchased from B.D.H. Chemicals. The potassium salt of protio-EPSP was prepared as described previously (*19*). All spectrophotometric and spectrofluorimetric measurements were obtained with a 1.0-cm path length. Deuteriated buffers were made up in 99.9% D_2O and the pD adjusted with KOD or DCl using a Russell combined pH/reference cell electrode coupled to an alpha 200 pH and conductivity meter at 25°C. pD values are quoted as pD = meter reading + 0.4.

Enzymes

N. crassa chorismate synthase was purified by the method of White *et al.* (*4*) and recombinant *E. coli* chorismate synthase was purified as reported previously (*19*). The *E. coli* protein concentration was estimated using ε_{280} = 21,440 M^{-1} cm^{-1} per subunit (*20*). Glycerol was added to substrate solutions to match the final glycerol concentration in enzyme solutions used for stopped-flow spectrophotometry/fluorimetry experiments to avoid mixing artefacts. All experiments with *E. coli* chorismate synthase were performed at 25°C and solutions were buffered using 50 mM Mops/KOH, pH 7.5, unless otherwise stated. One unit of enzyme is defined as the amount of enzyme required to convert 1 μmol of substrate to product in 1 min.

Chorismate Synthase Assays

N. crassa chorismate synthase was assayed either directly by uv spectrophotometry (*21*) or using a coupled spectrofluorimetric assay (*3*). The uv spectrophotometric assay involved monitoring the formation of chorismate at 275 nm (ε = 2630 $M^{-1}cm^{-1}$). Stock solutions of FMN (1 mM) and NADPH (2 mM) were freshly prepared on the day of each series of kinetic experiments and were stored in the dark on ice. Each assay was carried out in a 1-ml quartz cuvette containing NADPH (20 μM), FMN (10 μM), EPSP (50 μM), *N. crassa* chorismate synthase (typically 5 mU), Bis–Tris–HCl or triethanolamine–HCl buffer (50 mM, pH 7.0) and 50 mM KCl. Assays were initiated by the addition of NADPH. For consistent isotope effect measurements, stock solutions were prepared containing all the required ingredients except for NADPH. A total of nine determinations with EPSP and 15 with [4-^{2}H]EPSP were made. A kinetic isotope effect and its standard error was calculated from the means of the protio and deuterio data and their standard errors.

The fluorimetric assay of the *N. crassa* enzyme (*3*) involved coupling the production of chorismate with the production of anthranilate using anthranilate synthase. The formation of anthranilate was monitored using excitation and emission wavelengths of 313 and 390 nm, respectively. The fluorimeter was calibrated using anthranilate standards in Bis–Tris–HCl buffer (50 mM, pH 7.0). The detected fluorescence was not affected by the presence of either NADPH or FMN. Each assay was carried out in a 1-ml quartz fluorimeter cuvette containing NADPH (200 μM), FMN (10 μM), EPSP (20 μM), glutamine (10 mM), *N. crassa* chorismate synthase (0.2 mU), *N. crassa* anthranilate synthase (4 mU), Bis–Tris–HCl buffer (50 mM, pH 7.0), KCl

(50 mM), and $MgCl_2$ (5 mM). Ten times the NADPH concentration used in the UV assay was required to ensure it was saturating throughout the course of the assay due to the presence of a diaphorase activity in the partially purified anthranilate synthase preparation.

E. coli chorismate synthase was assayed directly by uv spectrophotometry as previously described (*22*) 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 the *E. coli* enzyme giving a final volume of 500 μl.

3-[2-$^2H_2$,4-^2H]Dehydroquinic Acid

3-Dehydroquinic acid, synthesized by the method of Grewe and Haendler (*23*), (100 mg, 0.53 mmol) was dissolved in deuterium oxide (D_2O, 7.5 ml) and the solution adjusted to pD of 9.5 with 4-(dimethylamino)pyridine (DMAP). The reaction mixture was left to exchange at room temperature for 7 days. The reaction was terminated when more than 95% exchange had occurred as seen by monitoring the disappearance of the C-4 hydrogen signal in the ^1H NMR spectrum. The reaction mixture was repeatedly washed with CH_2Cl_2 to remove the DMAP. The solution was adjusted to neutral pH and freeze dried. The product was used without further purification. δ_H (250 MHz, D_2O) 4.3 (residual signal, 4-*H*), 3.90 (1 H, dd, *J* 13.2, 5.8 Hz, 5-*H*), 2.53 (approx. 0.4 H, d, *J* 14.2 Hz, 2_{eq}-*H*), 2.30 (2 H, m, 6-H_2), other peaks were obscured by HOD suppression and signals arising from DMAP were 8.00 (2 H, d), 6.80 (2 H, d), and 3.10 (6 H, s). The relative percentage deuteriation at C-2_{ax}:C-2_{eq}:C-4 was 99:40:85%. δ_D (61.4 MHz, H_2O) 4.3 (1 D, broad s, 4-*D*), 3.1 (1 D, broad s, 2_{ax}-*D*), and 2.5 (approx. 0.6 D, broad s, 2_{eq}-*D*). *m/z* ES: 191 (M^--H) (2_{eq}-H is approx. 40% deuteriated and therefore $C_7H_8D_2O_6$ requiring 192 is expected to be the most common ion); CI: 211.1020 ($C_7H_{11}D_3O_6N^+$ requires 211.1006).

3-[4-^2H]Dehydroshikimic Acid

3-[2-$^2H_2$,4-^2H]Dehydroquinate (approx. 110 μmol) was dissolved in triply distilled MilliQ water (1.64 ml) and *E. coli* dehydroquinase type I (1.64 U) and *A. nidulans* dehydroquinase type II (1.97 U) were added to the solution. The reaction mixture was stirred at room temperature and the reaction monitored by ^1H NMR spectroscopy. Although the conversion to the product was complete within 2 h the reaction was left a further 5 days to ensure at least 85% removal of both the deuterium atoms at the C-2. The enzyme was removed by filtration through an Amicon Centricon 10. The product was used without further work up. δ_H (400 MHz, D_2O, pD 7.0) 6.35 (1 H, d, *J* 3.2 Hz, 2-*H*), 3.93 (1 H, dd, *J* 10.0, 5.3 Hz, 5-*H*), 3.03 (1 H, dd, *J* 18.3, 5.3 Hz, *pro-S* 6-*H*), 2.60 (1 H, ddd, *J* 18.3, 10.0, 3.2 Hz, *pro-R* 6-*H*) and signals arising from DMAP were 8.00 (2 H, d), 6.80 (2 H, d), and 3.10 (6 H, s). The residual percentage deuteriation at C-2 was 15 $\pm$ 3%.

[4-^2H]Shikimic Acid

To a final buffered solution of 3-[4-^2H]dehydroshikimate (12 mg, approx. 70 μmol) in triethanolamine (100 mM, pH 6.5) was added NADPH (52 mg, 75 μmol) and

shikimate dehydrogenase (12 U). The reaction mixture was stirred at room temperature for 24 h and the formation of shikimic acid was monitored by ^{1}H NMR spectroscopy. The reaction mixture was loaded directly onto a DEAE-Sephacel column (HCO_3^- form: 12 × 2 cm) at 1 ml min^{-1}, which was washed with 5 mM triethanolamine (20 ml) and eluted with a linear gradient of 5 to 500 mM triethanolamine (100 + 100 ml), collecting 3.5-ml fractions. Fractions were analyzed for shikimic acid by HPLC using a preparative Organic Acids column with an eluent of 50 mM HCO_2H. Fractions containing the product were pooled and lyophilized to yield a white solid. This was further purified by HPLC, to give [4-^{2}H]shikimic acid (9 mg, 51 μmol). δ_H (400 MHz, D_2O) 6.70 (1 H, dd, *J* 4.0, 1.6 Hz, 2-*H*), 4.40 (1 H, d, *J* 4.0 Hz, 3-*H*), 4.03 (1 H, dd, *J* 6.6, 5.3 Hz, 5-*H*), 2.75 (1 H, dd, *J* 18.0, 5.3 Hz, *pro-S* 6-*H*), 2.20 (1 H, ddd, *J* 18.0, 6.6, 2.0 Hz, *pro-R* 6-*H*). Extent of deuteriation by ^{1}H NMR approx. 90%. *m*/*z* ES: 174 (M^--H) ($C_7H_9O_5D$ requires 175); CI: 193.0935 ($C_7H_{13}O_5DN^+$ requires 193.0934), extent of deuteriation 85%.

5-Enolpyruvyl-[4-^{2}H]shikimate-3-phosphate ([4-^{2}H]EPSP)

A solution was prepared (1.08 ml) containing [4-^{2}H]shikimic acid (9 mg, 51 μmol), ATP (K^+ salt, 60 mM), phosphoenolpyruvate (PEP; K^+ salt, 60 mM), $MgCl_2$ (50 mM), and Tris−HCl buffer (300 mM, pH 7.5). Purified *E. coli* shikimate kinase (0.16 U) and EPSP synthase (0.08 U) were added, and the reaction mixture was incubated at 30°C for 24 h. Apyrase (Sigma grade VII, 1.6 U) and NaCl (~0.5 mg) were added, and the reaction mixture was incubated overnight at 30°C. After this treatment with apyrase to hydrolyze the ATP and ADP to AMP, the solution was loaded onto a Mono Q column and eluted with a linear gradient of 5 to 750 mM triethanolamine. Fractions were collected with continuous monitoring at 240 nm. EPSP eluted at 600 mM triethanolamine, and pooled fractions were lyophilized to give a highly deliquescent white solid, which, while still containing triethanolamine, was shown by chorismate synthase assay to contain 40 μmol EPSP (78%). HPLC retention time 9.0 min (semipreparative Organic Acids column 50 mM H_2SO_4, 0.6 ml min^{-1}). δ_H (D_2O, K^+ salt) 6.52 (1 H, broad s, 2-*H*), 5.22 (1 H, d, *J* 2.6 Hz, *pro-E* 8-*H*), 4.73 (1 H, d, *J* 2.6 Hz, *pro-Z* 8-*H*), 4.46 (1 H, dd, *J* 7.8, 4.4 Hz, 5-*H*), 3.02 (1 H, dd, *J* 17.8, 4.4 Hz, *pro-S* 6-*H*), 2.18 (1 H, dd, *J* 17.8, 7.8 Hz, *pro-R* 6-*H*), and the 3-H signal was obscured by HOD. Extent of C-4 deuteriation approx. 90%. δ_D (61.4 MHz, H_2O) 4.1 (1 D, s, 4-*D*). δ_P [101 MHz, D_2O, pD 8.0, $P(OMe)_3$] −136.3. *m*/*z* ES: 326 (M^+ + H).

Stopped-Flow Spectrophotometry

Single-turnover experiments were carried out using a Hi-Tech Scientific SF-61 DX2 stopped-flow spectrophotometer (Salisbury, UK) installed and operated in an anaerobic glove box with thermostatting of the observation cell ±0.1°C. The dead time of the instrument was measured to be 1.7 ms and only data after this time are shown with zero time, indicating the moment of mixing. Absorbance was monitored at 400 nm using a Xe lamp light source. Anaerobic substrate solution was mixed with anaerobic *E. coli* enzyme solution containing FMN that was reduced using dithionite. After mixing, the reaction mixtures contained substrate (20 μM), *E. coli* enzyme (30 μM), reduced FMN (30 μM), dithionite (1 mM) and buffer. Data from 1.7 to 945 ms after mixing were collected and analyzed. Kinetic data were fitted

using the Hi-Tech Scientific KinetAsyst 2.1 software. On one occasion, a total of five determinations with EPSP followed by five with [4-^{2}H]EPSP were made within 6 min. Kinetic isotope effects and their standard errors on the formation and decay of the flavin intermediate were calculated from the means of the protio and deuterio data and their standard errors. This was repeated on another occasion. The two independent determinations were combined to give the quoted values.

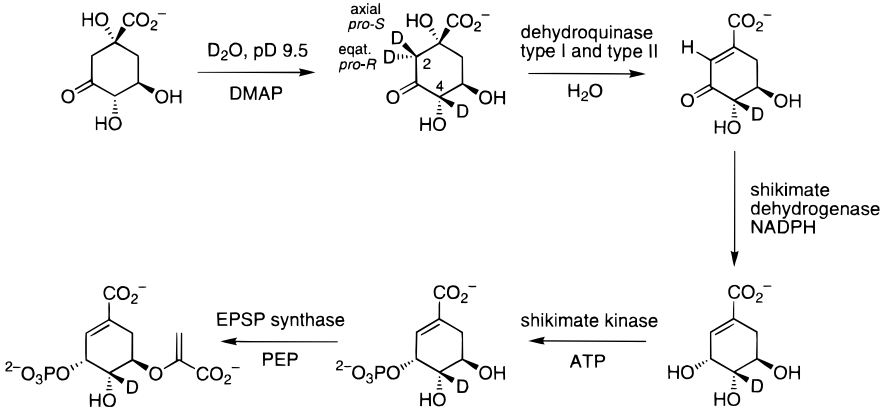
Stopped-Flow Spectrofluorimetry

Inorganic phosphate dissociation was measured in the presence of a fluorescent, *N*-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide-modified A197C mutant phosphate binding protein (MDCC-PBP) in real-time using stopped-flow spectrofluorimetry (*24*, *25*). Fluorescence associated with MDCC-PBP was monitored using a Hg light source, an excitation wavelength of 438 nm, and an emission filter with a 455-nm cut-off. Anaerobic substrate solution was mixed with anaerobic *E. coli* enzyme solution containing FMN that was reduced using dithionite. All solutions contained MDCC-PBP as well as a "phosphate mop," to remove adventitious inorganic phosphate prior to each experiment. The "phosphate mop" consisted of purine nucleoside phosphorylase and 7-methylguanosine. After mixing, the reaction mixtures contained substrate (4 μM), *E. coli* enzyme (6 μM), reduced FMN (6 μM), dithionite (500 μM), MDCC-PBP (12 μM), purine nucleoside phosphorylase (1 U ml^{-1}), 7-methylguanosine (1 mM), and buffer. Data from 1.7 to 466 ms after mixing were collected and analyzed. Kinetic isotope effects were determined and calculated as above.

RESULTS

Synthesis of [4-^{2}H]EPSP

The strategy used to synthesize EPSP labeled with deuterium specifically at C-4 is shown in Scheme 2. This involved the initial preparation of [4-^{2}H]shikimate,



SCHEME 2

which in turn was made from 3-dehydroquinate. Unlabeled 3-dehydroquinic acid was synthesized by the method of Grewe and Haendler (*23*). Base-catalyzed exchange of 3-dehydroquinate in D_2O resulted in introduction of deuterium not only at C-4, but also at both the axial *pro-S* and equatorial *pro-R* C-2 positions. A number of exchange conditions were explored, and in each case the axial C-2 position exchanged most rapidly, followed by the C-4 hydrogen. The best conditions found were at pD 9.5 using 4-(dimethylamino)pyridine (DMAP). The reaction was monitored by following the disappearance of the C-4 hydrogen signal in the ^1H NMR spectrum. Exchange took place slowly at room temperature over several days. The reaction was stopped when approximately 90% exchange had occurred. At this stage the extent of deuteriation was approximately 99, 40, and 85% at C-2_{ax}, C-2_{eq}, and C-4, respectively.

In order to study the effect of deuteriation at C-4 on the chorismate synthase reaction, it was essential that deuterium was only present at this position, and not at C-2. In order to remove the deuterium specifically from C-2 of dehydroquinate, the regiospecificity of dehydroquinase was exploited. At first sight, this does not appear a very promising strategy, since incubation of [2-$^2H_2$,4-^{2}H]dehydroquinate with type I dehydroquinase in water would lead to formation of an equilibrium mixture of (*6S*)-[2-^{2}H,4-^{2}H]dehydroquinate and [2-^{2}H,4-^{2}H]dehydroshikimate (1:15); the residual deuterium at C-2 being due to the stereospecific loss of deuterium from only the *pro-R* C-2 position in the type I dehydroquinase reaction. However, the type II dehydroquinase catalyzes the same conversion of 3-dehydroquinate to 3-dehydroshikimate, but by a different mechanism (*26*) and with stereospecific loss of the *pro-S* C-2 hydrogen (*27*). By incubating [2-$^2H_2$,4-^{2}H]dehydroquinate simultaneously with type I and type II dehydroquinase in water, all the deuterium at C-2 was exchanged. The reaction was followed by monitoring the increase in intensity of the C-2 hydrogen in the [4-^{2}H]dehydroshikimate produced. This product is not very stable and so was immediately reduced to [4-^{2}H]shikimate, using shikimate dehydrogenase and NADPH. The [4-^{2}H]shikimate was purified using two chromatographic steps. The deuterium was shown to be exclusively at C-4 by ^1H NMR spectroscopy, and the extent of deuteriation was determined to be 85% by mass spectrometry.

The [4-^{2}H]shikimate was then converted into [4-^{2}H]shikimate 3-phosphate using shikimate kinase and ATP. This was then converted into [4-^{2}H]EPSP using EPSP synthase and phosphoenolpyruvate (PEP). The presence of deuterium at C-4 in the final product was confirmed by ^1H and ^2H NMR spectroscopy and mass spectrometry. This synthetic approach using 6 steps from quinic acid to [4-^{2}H]EPSP compares favorably to an alternative from arabinose that would have required at least 12 (*28*).

^{4D}(*V*) Kinetic Isotope Effects with *N. crassa* and *E. coli* Chorismate Synthases

The chorismate synthases from *E. coli* and *N. crassa* have been studied most extensively. The *E. coli* enzyme has the advantage that it has been over-expressed as a recombinant enzyme (*19*), providing sufficient material for rapid kinetic and spectroscopic studies. On the other hand, the *N. crassa* enzyme has the advantage that it has an additional NADPH-dependent flavin reductase activity (*3, 4*), enabling it to be assayed without the need for chemical reductants under strictly anaerobic conditions. More detailed comparisons between the two enzymes have been made elsewhere (*19*).