RESEARCH COMMUNICATION

Escherichia coli chorismate synthase: a deuterium kinetic-isotope effect under single-turnover and steady-state conditions shows that a flavin intermediate forms before the C-(6*proR*)-H bond is cleaved

Stephen BORNEMANN,* Shankar BALASUBRAMANIAN,† John R. COGGINS,‡ Chris ABELL,† David J. LOWE* and Roger N. F. THORNELEY*§

*Nitrogen Fixation Laboratory, University of Sussex, Brighton, Sussex BN1 9RQ, †University Chemical Laboratory, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, and ‡Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

We report the observation of a deuterium kinetic isotope effect for the conversion of 5-enolpyruvylshikimate-3-phosphate into chorismate (${}^6\text{proR}^2\text{H}V = 1.13 \pm 0.03$) using recombinant chorismate synthase from *Escherichia coli*. Similar isotope effects were observed for the decay of a spectroscopically characterized flavin

intermediate (${}^6\text{proR}^2\text{H}k = 1.17 \pm 0.04$) during single-turnover experiments. The main rate-limiting steps and C-(6*proR*)-H bond breaking are therefore distinct and both must occur after the formation of the flavin intermediate and either before or concomitant with its decay.

INTRODUCTION

Chorismate synthase (EC 4.6.1.4) is present in bacteria, fungi and plants, but not in animals, and is therefore an attractive target for novel antibiotics and herbicides (Davies et al., 1994). It is the seventh enzyme of the shikimate pathway (Bentley, 1990; Haslam, 1993) and catalyses the conversion of 5-enolpyruvylshikimate-3-phosphate (EPSP) (compound 1, Scheme 1) into chorismate (compound 2, Scheme 1) in an overall *anti* 1,4-elimination of phosphate and the C-(6*proR*) hydrogen (Hill and Newkome, 1969; Onderka and Floss, 1969; Floss et al., 1972). Studies on model systems (Hill and Bock, 1978; Toromanoff, 1980) and molecular-orbital calculations (Fukui, 1965; Anh, 1968) suggest that the stereochemistry of the elimination disfavours a concerted process.

Several non-concerted mechanisms have been proposed for chorismate synthase, but there is no consensus as yet (Walsh et al., 1990). Proposed mechanisms include an X-group mechanism (Floss et al., 1972), an allylic rearrangement of phosphate followed by a 1,2-elimination (Ganem, 1978) and a stepwise loss of phosphate and a proton via a cationic intermediate (Bartlett et al., 1989; Hawkes et al., 1990). Reduced FMN is required for enzyme activity, despite there being no overall reduction or oxidation (Morell et al., 1967; Welch et al., 1974; White et al.,

1988; Ramjee et al., 1993). The exact role of flavin is not known, but transient u.v./visible spectroscopic changes during single-turnover experiments with the enzyme from *Escherichia coli* are consistent with a C(4a)-FMN adduct intermediate (Ramjee et al., 1991). In addition, the observation of a protein-bound flavin semiquinone in the presence of (6*R*)-6-fluoro-EPSP suggests the possibility of radical intermediates during normal turnover (Ramjee et al., 1992).

The cleavage of the non-activated C-(6*proR*)-H bond of EPSP might be expected to be a rate-limiting step of the chorismate synthase catalytic cycle. Indeed, moderate primary deuterium kinetic isotope effects of ${}^6\text{proR}^2\text{H}V = 2.7 \pm 0.2$ and ${}^6\text{proR}^2\text{H}(V/K) = 1.6 \pm 0.1$ have been reported for the *Neurospora crassa* chorismate synthase (Balasubramanian et al., 1990). The measurement of a pre-steady-state kinetic-isotope effect can give information about the timing of bond cleavage as well as its contribution to rate limitation. No pre-steady-state experiments are described for the *N. crassa* enzyme because adequate quantities cannot be isolated easily. The recombinant *E. coli* enzyme can, however, be purified on a scale sufficient for single-turnover experiments (Ramjee, 1992).

The enzymes isolated from *E. coli* and *N. crassa* have the same requirement for reduced flavin for activity (Morell et al., 1967; Welch et al., 1974; White et al., 1988), a shared preference for FMN over FAD (Schaller et al., 1991), comparable K_m values for EPSP [$1.3 \mu\text{M}$ (Ramjee et al., 1994) and $2.2\text{--}2.7 \mu\text{M}$ (Bartlett et al., 1986; Balasubramanian et al., 1990), respectively], similar pH optima [$7.5\text{--}8.0$ (Ramjee et al., 1994) and 8.1 (Schaller et al., 1991) respectively] and α_4 quaternary structures (White et al., 1988). A conspicuous difference between the two is that the *E. coli* enzyme [subunit $M_r = 39000$ (Charles et al., 1990)] is monofunctional (White et al., 1988), whereas the somewhat larger *N. crassa* enzyme [subunit $M_r = 50000$ (White et al., 1988)] is bifunctional, having an additional NADPH-dependent flavin reductase (diaphorase) activity (Welch et al., 1974; White et al., 1988). In the present paper we report the observation of a

Scheme 1

Abbreviations used: EPSP, 5-enolpyruvylshikimate-3-phosphate; the kinetic-isotope-effect nomenclature is that given in Northrop (1975).

§ To whom correspondence should be sent.

small (*6proR*)-deuterium kinetic isotope effect during single-turnover and steady-state experiments using recombinant chorismate synthase from *E. coli*.

EXPERIMENTAL

All chemicals and biochemicals were of the highest grade available and, unless otherwise stated, were purchased from Sigma, Poole, Dorset, U.K. Sodium dithionite was purchased from BDH, Poole, Dorset, U.K. The potassium salt of EPSP was prepared essentially as described previously (Knowles et al., 1970). Solutions were buffered using 50 mM Mops/KOH, pH 7.5. The dibarium salts of (6*R*)-[6-²H]EPSP (80% isotopic purity, the remainder being the protio form) and (6*S*)-[6-²H]EPSP (80% isotopic purity, of which 90% was 6*S*, the remainder being the protio form) were prepared as described previously (Balasubramanian and Abell, 1991) and converted into the soluble potassium salts before use. The spectrophotometric measurements were obtained with a 1 cm-path-length cell.

Recombinant *E. coli* chorismate synthase was purified to >95% purity as judged by SDS/PAGE from an overproducing strain of *E. coli* (AB2849/pGM605) by the method of White et al. (1988) with the modifications described by Ramjee (1992).

The formation of the product diene ($\epsilon_{275} = 2630 \text{ M}^{-1} \cdot \text{cm}^{-1}$) was monitored at 25 °C as previously described (Ramjee et al., 1994) and substrate solutions were standardized using this method. Anaerobic assay mixtures contained substrate (50 μM), FMN (10 μM), potassium oxalate (1 mM) and Mops buffer, pH 7.5. The flavin was photoreduced before the addition of the *E. coli* enzyme (2.2 μg) giving a final volume of 500 μl .

Single-turnover experiments were carried out using a Hi-Tech Scientific SF-51 stopped-flow spectrophotometer installed and operated in an anaerobic glove box as described previously (Thorneley and Lowe, 1983). Substrate solution was mixed with enzyme and reduced FMN solution to give a final anaerobic reaction mixture containing, unless otherwise stated, substrate (20 μM), enzyme (30 μM), reduced FMN (30 μM), dithionite (1 mM) and Mops buffer, pH 7.5 at 25 °C. 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 IS-2 v2.3b5 software.

RESULTS AND DISCUSSION

The isotope effect on V_{max} with the *E. coli* enzyme using (6*R*)-[6-²H]EPSP was determined by monitoring the formation of the product diene at 275 nm under anaerobic conditions (Figure 1). The initial concentration of 50 μM for both the labelled and unlabelled substrates was well above the $K_{\text{m, EPSP}}$ of $1.3 \pm 0.3 \mu\text{M}$, giving progress curves that were linear for more than 90% of their duration. An analysis of quadruplicate experiments gave ${}^{6\text{proR}^2\text{H}}V = 1.13 \pm 0.03$. The correspondingly small effect on ${}^{6\text{proR}^2\text{H}}(V/K)$ was not discernible within the accuracy of the K_{m} determination. The (6*R*)-[6-²H]EPSP was 80% deuteriated, with the remainder being the protio form. The maximum theoretical (Northrop, 1975) isotope effect on V_{max} for this reaction with 100% isotopically pure deuteriated substrate is 1.16 ± 0.03 . Within experimental error, no isotope effect on V_{max} was observed using (6*S*)-[6-²H]EPSP (${}^{6\text{proS}^2\text{H}}V = 1.01 \pm 0.03$; results not shown). Assuming the isotope effect is a primary effect this result confirms that the *E. coli* enzyme catalyses the formation of chorismate with *anti* stereochemistry (Hill and Newkome, 1969; Floss et al., 1972).

Figure 1 Kinetic-isotope effect observed during steady-state conditions

The formation of the diene product chorismate ($\epsilon_{275} = 2630 \text{ M}^{-1} \cdot \text{cm}^{-1}$) was monitored at 275 nm. Anaerobic reaction mixtures contained substrate (50 μM), FMN (10 μM), potassium oxalate (1 mM) and Mops buffer, pH 7.5 at 25 °C. The flavin was photoreduced before the addition of the *E. coli* enzyme (2.2 μg) giving a final volume of 500 μl . (a) EPSP; (b) (6*R*)-[6-²H]EPSP.

Figure 2 Kinetic-isotope effect observed during single-turnover conditions

The formation and decay of the flavin intermediate during a single-turnover stopped-flow experiment was monitored at 400 nm (see Figure 3). The final anaerobic reaction mixtures contained substrate (20 μM), *E. coli* enzyme (30 μM), FMN (30 μM), dithionite (1 mM) and Mops buffer, pH 7.5 at 25 °C. Top panel: curve a, EPSP; curve b, (6*R*)-[6-²H]EPSP. The smooth overlaid curves were fitted to the observed data using two first-order rate constants describing the formation and subsequent decay of the intermediate: curve a, 160 s^{-1} and 52 s^{-1} ; curve b, 160 s^{-1} and 44 s^{-1} . Bottom panel: the residuals for these fits are depicted as curves a₁ and b₁ respectively. The residuals of fits where the second exponent was constrained at 48 s^{-1} show a clear deterioration: curve a₂, 185 s^{-1} and 48 s^{-1} ; curve b₂, 140 s^{-1} and 48 s^{-1} .

The isotope effect using (6*R*)-[6-²H]EPSP on the kinetics of the flavin intermediate during single-turnover experiments is best explained by a decrease in its rate of decay rather than any change in its rate of formation (Figure 2). Fitting the data obtained with EPSP and (6*R*)-[6-²H]EPSP using two first-order

Figure 3 Difference spectra of the transient flavin intermediate relative to reduced flavoenzyme

The difference spectra of the transient flavin intermediate with EPSP (—) and (6*R*)-[6-²H]EPSP (---) are essentially coincident. The data were obtained after 2 ms using a rapid-scanning photodiode-array stopped-flow spectrophotometer. The anaerobic reaction mixtures contained substrate (14 μ M), *E. coli* enzyme (20 μ M), FMN (40 μ M), dithionite (1 mM) and Mops buffer pH 7.5 at 25 °C. The blank spectrum was obtained from a control experiment without substrate. The arrow indicates the isosbestic point at 359 nm.

exponentials gave identical values for its rate of formation (160 s⁻¹) and different values for its rate of decay (52 and 44 s⁻¹ respectively). Fitting the data with the second exponent constrained at an intermediate value (48 s⁻¹) showed a clear deterioration in the fits. The isotope effect on the exponential decay of the intermediate from quadruplicate experiments gave $^{2\text{H}}k = 1.17 \pm 0.04$. The same value was obtained from multiple-turnover experiments (results not shown). Similar isotope effects on V_{max} and the decay of the intermediate were observed when the reaction mixtures were buffered at pH 7.0 (50 mM KH₂PO₄/KOH; results not shown) rather than pH 7.5 (50 mM Mops). The isotope effect for this reaction with 100% isotopically pure deuterated substrate is estimated to be 1.20 ± 0.05 .

The high-resolution difference spectra of the transient flavin intermediate observed with the photodiode-array stopped-flow spectrophotometer were essentially identical for the protio and deuterio substrates (Figure 3). The spectra are also consistent with a lower-resolution spectrum obtained from single-wavelength stopped-flow amplitude data (Ramjee et al., 1991). The spectrum did not change, other than in intensity, as the flavin intermediate formed and decayed (results not shown), and an isosbestic point was identified at 359 nm with both substrates. Deuteration at the 6*R* position therefore had no effect on the nature of the observed intermediate other than the small kinetic isotope effect described above.

The small isotope effects described here strongly suggest that C-(6*proR*)-H bond breaking does not contribute significantly to the overall rate-limiting steps of the *E. coli* chorismate synthase reaction (Northrop, 1975). This is in contrast with the moderate isotope effects observed with the *N. crassa* enzyme (Balasubramanian et al., 1990), which are typical for enzymic reactions where the carbon-hydrogen bond-breaking step is partially rate-limiting (Northrop, 1975). The two enzymes clearly have different rate-limiting steps, but this alone does not imply that they have different mechanisms.

If the mechanism of the *E. coli* enzyme involves the loss of phosphate before C-(6*proR*)-H bond cleavage, the C-6-H₂ σ bonds should contribute to the stabilization of the allylic carbocation intermediate through hyperconjugation. The $^{6\text{proR}^2\text{H}}V$ effect may therefore be a secondary vinylogous β -

Scheme 2

deuterium effect, the magnitude of which suggests full expression. The inverse inductive component of such a secondary effect is likely to be negligible given the through-bond distance between C-3 and C-6. The hyperconjugative component of the secondary vinylogous β -effect would be maximal when the C-(6*proR*)-H σ orbital and the p-orbital of the adjacent vinyl system are eclipsed. If the effect is secondary, the conformation of the cyclic substrate in the active site of the enzyme must cause the (6*proR*)-H to be pseudo-axial because $^{6\text{proR}^2\text{H}}V$ was significantly greater than $^{6\text{proS}^2\text{H}}V$ [see the analogous example of neuraminidase (Guo and Sinnott, 1993)]. The ring system could then adopt a half-chair conformation (Scheme 2) at the active site of the enzyme with the phosphate leaving group in a pseudo-axial conformation facilitating C-3-O bond cleavage. This conformation is indeed adopted in solution by shikimate-3-phosphate and the 4,5-pyruvylketal of EPSP (Castellino et al., 1991). If the observed isotope effect is a secondary vinylogous β -deuterium effect, the substrate would appear not to undergo a conformational change on binding to the *E. coli* enzyme. An isotope effect on $^{4\text{H}}V$ would confirm this hypothesis. An additional consequence of this interpretation of the *E. coli* isotope effect is that the mechanism of the enzyme from *N. crassa* must be different because its $^{6\text{proR}^2\text{H}}V$ effect is clearly primary.

The mere observation of the flavin intermediate with the *E. coli* enzyme clearly means that its formation cannot be significantly rate-limiting. Importantly, the similar isotope effects on the decay of the intermediate and the V_{max} suggest that steps subsequent to the decay of the flavin intermediate, which may include product release for example, do not contribute significantly to rate-limitation. If the isotope effect is primary, it must be expressed on C-(6*proR*)-H bond cleavage, and if it is secondary, it must be expressed prior to C-(6*proR*)-H bond cleavage. One can therefore conclude that the main rate-limiting steps and C-(6*proR*)-H bond breaking are distinct and that both must occur after the formation of the flavin intermediate and either before or concomitant with its decay. The lack of a large initial burst of phosphate with the *E. coli* enzyme using a rapid chemical quench technique (Hawkes et al., 1990) is consistent with phosphate cleavage occurring after the formation of the flavin intermediate. It is not yet clear whether C-(6*proR*)-H bond cleavage or phosphate cleavage occurs first, but both events appear to occur after the formation of the flavin intermediate and a concerted process cannot be ruled out.

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REFERENCES

- Anh, N. T. (1968) *J. Chem. Soc. Chem. Commun.*, 1089–1090
- Balasubramanian, S. and Abell, C. (1991) *Tetrahedron Lett.* **963**–966
- Balasubramanian, S., Abell, C. and Coggins, J. R. (1990) *J. Am. Chem. Soc.* **112**, 8581–8583
- Bartlett, P. A., Maitra, U. and Chouinard, P. M. (1986) *J. Am. Chem. Soc.* **108**, 8068–8071
- Bartlett, P. A., McLaren, K. L., Alberg, D. G., Fässler, A., Nyfelder, R., Lauhon, C. T. and Grissom, C. B. (1989) in *Proceedings of the Society for Chemical Industry Pesticides Group Meeting, BCPC Monograph 42*, pp. 155–170, Lavenham Press, Lavenham, Ipswich
- Bentley, R. (1990) *Crit. Rev. Biochem. Mol. Biol.* **25**, 307–384
- Castellino, S., Leo, G. C., Sammons, R. D. and Sikorski, J. A. (1991) *J. Org. Chem.* **56**, 5176–5181
- Charles, I. G., Lamb, H. K., Pickard, D., Dougan, G. and Hawkins, A. R. (1990) *J. Gen. Microbiol.* **136**, 353–358
- 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) *Antimicrob. Agents Chemother.* **38**, 403–406
- Floss, H. G., Onderka, D. K. and Carroll, M. (1972) *J. Biol. Chem.* **247**, 736–744
- Fukui, K. (1965) *Tetrahedron Lett.* 2427–2432
- Ganem, B. (1978) *Tetrahedron* **34**, 3353–3383
- Guo, X. and Sinnott, M. L. (1993) *Biochem. J.* **294**, 653–656
- Haslam, E. (1993) in *Shikimic Acid: Metabolism and Metabolites*, John Wiley and Sons, Chichester
- Hawkes, T. R., Lewis, T., Coggins, J. R., Mousdale, D. M., Lowe, D. J. and Thorneley, R. N. F. (1990) *Biochem. J.* **265**, 899–902
- Hill, R. K. and Bock, M. G. (1978) *J. Am. Chem. Soc.* **100**, 637–639
- Hill, R. K. and Newkome, G. R. (1969) *J. Am. Chem. Soc.* **91**, 5893–5894
- Knowles, P. F., Levin, J. G. and Sprinson, D. B. (1970) *Methods Enzymol.* **17**, 360–362
- Morell, H., Clark, M. J., Knowles, P. F. and Sprinson, D. B. (1967) *J. Biol. Chem.* **242**, 82–90
- Northrop, D. B. (1975) *Biochemistry* **14**, 2644–2651
- Onderka, D. K. and Floss, H. G. (1969) *J. Am. Chem. Soc.* **91**, 5894–5896
- Ramjee, M. K. (1992) Ph.D. Thesis, University of Sussex
- Ramjee, M. N., Coggins, J. R., Hawkes, T. R., Lowe, D. J. and Thorneley, R. N. F. (1991) *J. Am. Chem. Soc.* **113**, 8566–8567
- Ramjee, M. N., Balasubramanian, S., Abell, C., Coggins, J. R., Davies, G. M., Hawkes, T. R., Lowe, D. J. and Thorneley, R. N. F. (1992) *J. Am. Chem. Soc.* **114**, 3151–3153
- Ramjee, M. K., Coggins, J. R., Hawkes, T. R., Lowe, D. J. and Thorneley, R. N. F. (1993) *Bioorg. Med. Chem. Lett.* **3**, 1409–1414
- Ramjee, M. K., Coggins, J. R. and Thorneley, R. N. F. (1994) *Analytical Biochem.* **220**, 137–141
- Schaller, A., van Afferden, M., Windhofer, V., Bülow, S., Abel, G., Schmid, J. and Amrhein, N. (1991) *Plant Physiol.* **97**, 1271–1279
- Thorneley, R. N. F. and Lowe, D. J. (1983) *Biochem. J.* **215**, 393–403
- Toromanoff, E. (1980) *C. R. Seances Acad. Sci. Ser. C* **290**, 81–84
- Walsh, C. T., Liu, J., Rusnak, F. and Sakaitani, M. (1990) *Chem. Rev.* **90**, 1105–1129
- Welch, G. R., Cole, K. W. and Gaertner, F. H. (1974) *Arch. Biochem. Biophys.* **165**, 505–518
- White, P. J., Millar, G. and Coggins, J. R. (1988) *Biochem. J.* **251**, 313–322

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