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Chorismate synthase

Pre-steady-state kinetics of phosphate release from 5-enolpyruvylshikimate 3-phosphate

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The pre-steady-state kinetics of phosphate formation from 5-enolpyruvylshikimate 3-phosphate catalysed by *Escherichia coli* chorismate synthase (EC 4.6.1.4) were studied by a rapid-acid-quench technique at 25 °C at pH 7.5. No pre-steady-state 'burst' or 'lag' phase was observed, showing that phosphate is released concomitant with the rate-limiting step of the enzyme. The implications of this result for the mechanism of action of chorismate synthase are discussed.

INTRODUCTION

The shikimate pathway of plants is an attractive target for herbicides, since this route to the biosynthesis of a wide range of primary and secondary metabolites including aromatic amino acids, folate, ubiquinone and lignin is not present in animals; 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase is inhibited by the herbicide glyphosate [*N*-(phosphonomethyl)glycine] (Steinrücken & Amrhein, 1980). An alternative site of action for new herbicides is the final enzyme in the pathway, chorismate synthase (EC 4.6.1.4), which catalyses the conversion of EPSP into chorismate (Scheme 1). Chorismate synthase (M_r 38000) surprisingly requires a reduced flavin cofactor for activity, although the conversion of substrate into product does not involve any overall oxidation or reduction (White *et al.*, 1988). The *trans* stereochemistry of the 1,4-elimination of a proton and phosphate ion from EPSP (Hill & Newkome, 1969) is unexpected, since both theoretical and experimental models favour *cis* geometry for a concerted elimination reaction (Hill & Bock, 1978). Therefore mechanisms involving two steps have been favoured previously in order to rationalize the *trans* stereochemistry. Mechanisms involving the initial elimination of phosphate to form either a carbonium ion intermediate or an intermediate covalently bound to the enzyme (Floss *et al.*, 1972) are discussed below. An alternative mechanism involving an allylic 1,3-shift of the phosphate group is unlikely, since the putative intermediate, iso-EPSP, is a good competitive inhibitor (K_i 8.7 μM for the *Neurospora crassa* enzyme) but not an alternative substrate (cf. K_m 2.7 μM for EPSP) (Bartlett *et al.*, 1986). We report here the pre-steady-state kinetics of phosphate release from functioning chorismate synthase. The data are discussed in terms of the mechanisms outlined above.

MATERIALS AND METHODS

The purification of *Escherichia coli* chorismate synthase from an overproducing strain of *E. coli*, the purification of EPSP and the enzyme assay have been detailed previously (White *et al.*, 1988; Coggins *et al.*, 1987). Chorismate synthase was better than 95% pure as judged by SDS/PAGE; the barium salt of EPSP was 80% pure on the basis of enzyme-releasable phosphate. Biochemicals were of the highest purity available from Sigma Chemical Co. (Poole, Dorset, U.K.). The apparatus for studying quenched-flow enzyme kinetics under anaerobic conditions at 25 °C has been described elsewhere (Thorneley & Lowe, 1985).

Two Hamilton gas-tight syringes (2.5 ml) were filled with equal volumes of 50 mM-Tris/HCl buffer, pH 7.5, with $Na_2S_2O_4$ (2 mM) containing in one case EPSP (180 μM) and in the other chorismate synthase (2 mg/ml) and FMN (35 μM). Each datum point in Fig. 1 corresponds to a single 'shot' in which 0.2 ml of solution from each syringe was rapidly mixed and after a defined reaction time quenched with 0.4 ml of ice-cooled 1.0 M-$HClO_4$. Precipitated protein was removed from the quenched sample by spinning for 5 min in an Eppendorf centrifuge at 5 °C. The supernatant (0.75 ml) was neutralized with 0.37 ml of 1.0 M-NaOH and then freeze-dried overnight to reduce the volume. The phosphate in each sample was determined by the molybdate/Malachite Green method (Lanzetta *et al.*, 1979), which was modified as follows. Triton N101 was used instead of Sterox detergent, freeze-dried samples were dissolved in the molybdate/Malachite Green reagent (1.5 ml) and after 3 min 1.56 M-citric acid (0.3 ml) was added. These reagent volumes were doubled when more than 30 nmol of phosphate was expected in the sample. Phosphate added as an internal standard was recovered quantitatively with no significant hydrolysis of EPSP.

The concentration of chorismate synthase was determined by the biuret method, with BSA as a standard.

RESULTS AND DISCUSSION

Fig. 1 shows the kinetics of phosphate release from

Abbreviation used: EPSP, 5-enolpyruvylshikimate 3-phosphate [O^5-(1-carboxyvinyl)-3-phosphoshikimate].

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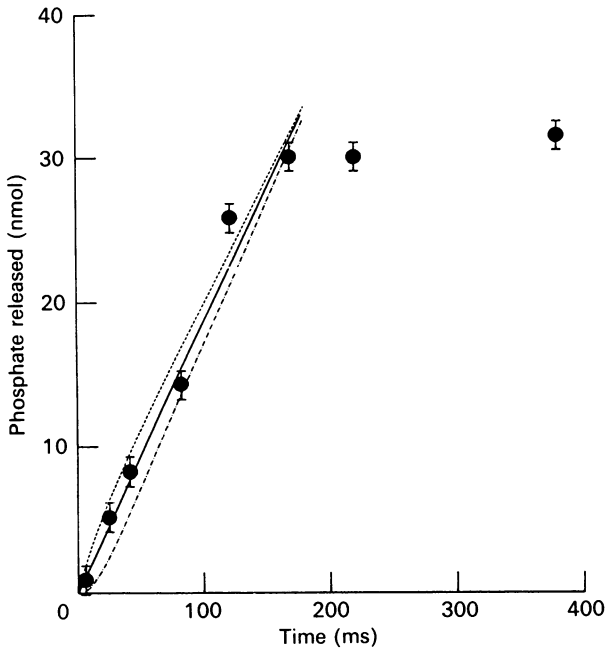


Fig. 1. Pre-steady-state phosphate release from EPSP

The data were obtained with the rapid-quench technique as described in the Materials and methods section. A reagent blank of 10.2 nmol was substracted from each point. The continuous line is a best-fit straight line through the data up to 200 ms, after which time the substrate, EPSP, is exhaused. The sharp decrease in rate is entirely consistent with the $K_m < 10\ \mu M$ for EPSP. The error bars represent the standard deviation of the determination of phosphate. The two broken lines represent limiting cases for a two-step mechanism in which phosphate is released either in the first step, k_1 (upper line), or second step, k_2 (lower line), i.e.:

$$E+S \xrightarrow{\text{Fast}} ES \xrightarrow{k_1} E'S \xrightarrow{k_2} E'P \xrightarrow{\text{Fast}} E+P$$

using the analytical solutions of the related differential equations:

$$\text{Total phosphate (upper line)} = \frac{[A]k_1}{(1+K)}\left(Kt-\frac{1}{a}-e^{-at}\right)$$

and

$$\text{Total phosphate (lower line)} = \frac{[A]k_2}{(1+K)}\left(t-\frac{1}{a}-e^{-at}\right)$$

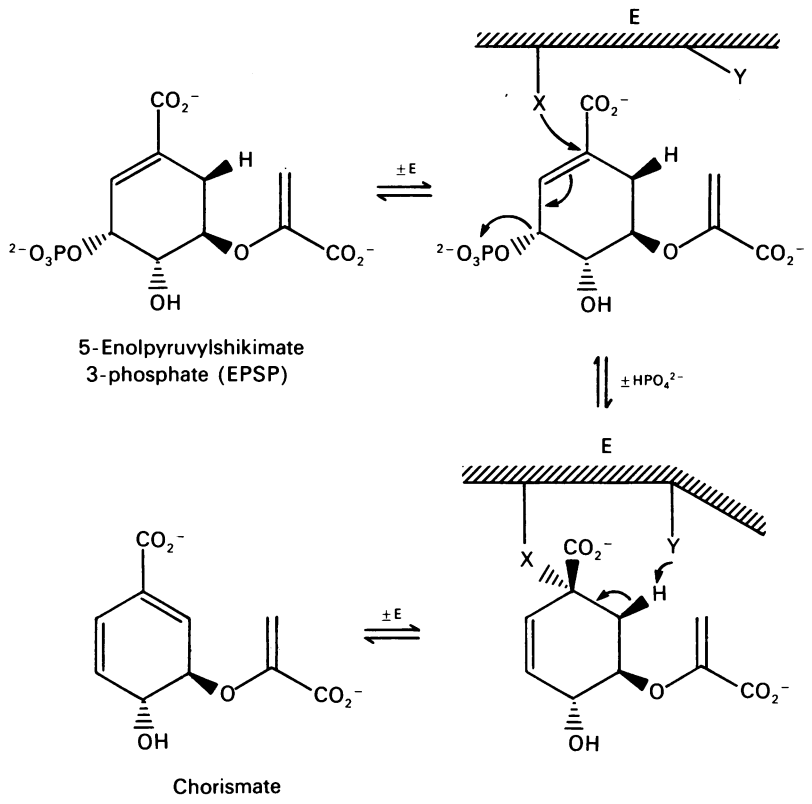
where [A] is the total active-site concentration, $K = k_2/k_1$ and $a = k_1+k_2$. Both simulations assume that each step occurs with the same rate constant ($k_1 = k_2 = 34\ s^{-1}$ for the upper line, $k_1 = k_2 = 40\ s^{-1}$ for the lower line), which is about twice the value of $k_{cat.} = 18\ s^{-1}$ obtained from the straight line through the data points.

EPSP catalysed by chorismate synthase. The time course is linear up to approx. 150 ms, after which time a decrease in rate attributable to EPSP limitation occurs ($K_m < 10\ \mu M$). There is no detectable 'lag' or 'burst' phase preceding the steady-state release of phosphate. The gradient of the line in Fig. 1 corresponds to a specific activity of 400 nmol of P_i released/s per mg of protein. This gives a turnover time of 55 ms assuming one active site per monomer of M_r 38000 (White *et al.*, 1988). Under the conditions used to obtain the data in Fig. 1, if phosphate had been released in an essentially irreversible rapid reaction preceding the rate-determining step, an easily detectable 'burst' of 10 nmol of phosphate would have occurred. The absence of a 'burst' phase causes us to conclude that phosphate is most probably released concomitantly with the rate-limiting step. This conclusion rests on the assumption that the total enzyme monomer concentration reflects the active-site concentration. This assumption is justified, since only 50% of the total units of activity were lost during purification to homogeneity (White *et al.*, 1988). We would have detected a 'burst' even if the enzyme had been only 20% active (detection limit 2 nmol of phosphate in Fig. 1). However, the simulations in Fig. 1 show that the quality of the data does not allow us to exclude a two-step mechanism in which P_i is released either in the first step (small 'burst' predicted) or in the second step (small 'lag' predicted), provided that both steps occur with rate constants of similar magnitude (approx. 36 s^{-1}), i.e. twice the catalytic-centre activity of approx. 18 s^{-1}).

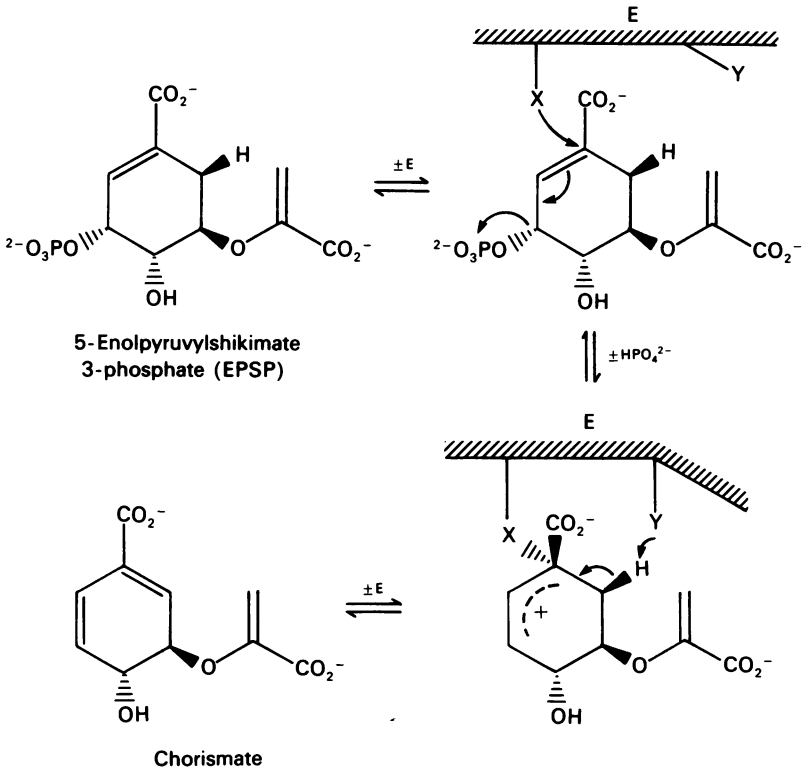
Our failure to observe a phosphate 'burst' on acid quenching shows not only that at pH 7.5 phosphate is released concomitantly with the rate-limiting step but also that EPSP is not bound, before the rate-limiting step, in a form that yields phosphate on quenching with acid. Consistent with this conclusion, when enzyme (2.6 nmol) containing oxidized FMN, in the absence of $Na_2S_2O_4$, was incubated with EPSP (90 μM) for 15 min and then acid-quenched, no phosphate was detected (the limit of detection was 0.2 nmol of phosphate corresponding to 8% of the enzyme monomer concentration).

The specific activity of the chorismate synthase increased with increasing enzyme concentration at a constant substrate concentration ($K_i < 10\ \mu M$, EPSP concentration 90 μM). At enzyme concentration < 25, 100 and 1000 μg/ml specific activities of 50, 150 and 400 nmol of P_i released/s per mg were measured. We therefore suggest that over this range of protein concentrations there is a change in quaternary structure of the enzyme that affects activity. A detailed analysis of this effect is beyond the scope of the current investigation. However, the mechanistic conclusions of this paper depend not on the state of the enzyme at a particular concentration but only on a measured steady-state rate of P_i release under the conditions of the rapid-quench experiments when no 'lag' or 'burst' phases were detected.

Floss *et al.* (1972) have shown that the stereochemistry of 1,4-conjugate elimination of phosphoric acid from EPSP is anti and concluded that a concerted mechanism was unlikely. They therefore proposed a two-step mechanism (Scheme 1) in which a nucleophilic group X on the enzyme forms a covalent bond with C-1 and displaces the phosphate in a syn S_N2' process. Group X is subsequently removed in an anti E2 reaction involving the removal of the *pro-R* proton from C-6 by another basic group Y. The absence of a pre-steady-state burst of phosphate release (Fig. 1) shows that, if Scheme 1 describes the mechanism, then the rate of the initial release of phosphate must be the same as or lower than that of the elimination of group X. In this case X would need to be a similar or better leaving group than phosphate and eliminate via an E_1 rather than an E_2 mechanism (the *pro-R* proton on C-6 is too weakly acidic for an E_2 process to be fast). It is difficult to suggest a candidate for X that



Scheme 1. Mechanism of action of chorismate synthase involving a nucleophilic group X and base Y at the enzyme active site as originally proposed by Floss *et al.* (1972)



Scheme 2. Mechanism of action of chorismate synthase involving a carbonium ion intermediate stabilized by charge interactions at the enzyme active site

Removal of a proton at C-6 by a basic group Y generates the enzyme–product complex.

would be both sufficiently nucleophilic to displace phosphate and at the same time be an equally good or better leaving group than phosphate. Bartlett *et al.* (1986) observed that iso-EPSP, in which the phosphate group is present in C-1, is a good competitive inhibitor of EPSP. If a group X is involved in EPSP binding and activation, the phosphate group at C-1 of iso-EPSP would be expected to interact unfavourably with group X, thereby greatly decreasing the affinity of the enzyme for this inhibitor. Therefore the K_i of 8.7 μM for iso-EPSP when compared with the K_m of 2.7 μM for EPSP (*N. crassa* enzyme) suggests that a group X is not present at the active site and provides further evidence against the mechanism shown in Scheme 1.

A simpler mechanism shown in Scheme 2, involving a carbonium ion intermediate, is entirely consistent with the data in Fig. 1. The elimination of a proton from position C-1 of the carbonium ion should be much faster than the loss of phosphate that generates this intermediate. Similar mechanisms involving the stabilization of carbonium or oxonium ion intermediates have been proposed for isopentyl pyrophosphate isomerase (Cornforth, 1973), glycosidases (Lalegerie *et al.*, 1982), EPSP synthase (Anton *et al.*, 1983) and squalene synthase (Sandifer *et al.*, 1982).

A third possibility, also consistent with the data in Fig. 1, is that when EPSP binds to chorismate synthase the ring is distorted so as to favour a concerted *trans*-1,4-elimination without either the involvement of a covalent bond to group X (Scheme 1) or the formation of a transient carbonium ion (Scheme 2). Although *cis*-elimination is preferred on theoretical grounds, chemical model studies (Hill & Bock, 1978) were equivocal. It is possible that activation energy differences between *cis*- and *trans*-elimination processes are small and that only a small distortion of the EPSP ring would be necessary to induce the observed *trans* stereochemistry. The carbonium ion mechanism of Scheme 2 is, of course, a limiting case of such a distortion.

We have shown that phosphate is released in the rate-determining step of chorismate synthase. It therefore becomes important to determine the kinetic isotope effect when the *pro-R* ^{1}H on C-6 is replaced by ^{2}H. A pronounced effect would favour the concerted *trans* process, no effect the carbonium ion intermediate. In addition, our failure to observe a pre-steady-state burst for phosphate release places constraints on any proposed mechanism and allows us to exclude a Floss *et al.* (1972) 'X group mechanism' in which phosphate is eliminated faster from EPSP than chorismate is released from the enzyme.

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