Some kinetic properties of the tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from *Neurospora crassa*

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The steady-state kinetic properties of purified tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from *Neurospora crassa* were examined. The results suggest that the enzyme obeys a Rapid-Equilibrium Ordered mechanism, in which phosphoenolpyruvate is the first substrate to bind and 3-deoxy-D-arabino-heptulosonate 7-phosphate is the second product to be released, rather than a Ping Pong mechanism as has been reported previously. The inhibition by tryptophan was found to be parabolic competitive with respect to D-erythrose 4-phosphate and parabolic non-competitive with respect to phosphoenolpyruvate. The enzyme was inactivated by EDTA, and could be protected against this inactivation by phosphoenolpyruvate or 3-deoxy-D-arabino-heptulosonate 7-phosphate but not by D-erythrose 4-phosphate, tryptophan or P_i . This suggests that the enzyme may be a metalloenzyme.

3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase [7-phospho-2-keto-3-deoxy-D-arabino-heptonate D-erythrose 4-phosphate-lyase (pyruvate-phosphorylating), EC 4.1.2.15] catalyses the formation of 3-deoxy-D-arabino-heptulosonate 7-phosphate and P_i from phosphoenolpyruvate and D-erythrose 4-phosphate. This is the first committed step of the pathway by which the three aromatic amino acids, tyrosine, tryptophan and phenylalanine, are synthesized. The enzyme represents an important site for the regulation of the pathway, and *Neurospora crassa* contains three isoenzymes of the synthase, each of which is subject to feedback inhibition by one of the three aromatic amino acids (Jensen & Nasser, 1968; Hoffmann *et al.*, 1972).

In a large number of cases the steady-state kinetics of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases have been examined, and parallel kinetic patterns indicative of a Ping Pong mechanism (Cleland, 1970) with an enzyme-bound enolpyruvyl intermediate were found (see, e.g., Eberpächer *et al.*, 1970; Görisch & Lingens, 1971). In these studies an insensitive discontinuous assay and a restricted range of substrate concentrations were used. In a few cases conflicting results have been obtained, and a re-examination of the kinetics with a more suitable range of substrate concentrations has yielded intersecting kinetic patterns, and a Sequential Ordered mechanism (Cleland, 1970) has been proposed with phosphoenolpyruvate as the first substrate to bind and 3-deoxy-D-arabino-heptulosonate 7-phosphate as the second product to be released.

Thus Staub & Dénes (1969a) obtained parallel kinetics for the phenylalanine-sensitive isoenzyme from *Escherichia coli*, whereas Simpson & Davidson (1976) subsequently obtained intersecting kinetics for the same enzyme. Similarly, early studies of the tyrosine-sensitive isoenzyme from *Salmonella typhimurium* yielded parallel kinetics (De Leo & Sprinson, 1968; Nagano & Zalkin, 1970), whereas De Leo *et al.* (1973) obtained intersecting kinetics, and Moldovanyi & Dénes (1968) obtained parallel kinetics for the tyrosine-sensitive isoenzyme from *E. coli*, whereas Schoner & Herrmann (1976) obtained intersecting kinetics. The kinetics of the partially purified tryptophan-sensitive isoenzyme from *N. crassa* have been examined by using a discontinuous assay, and a Ping Pong mechanism was proposed for the enzyme (Ip & Doy, 1979). This isoenzyme has now been purified to homogeneity (Nimmo & Coggins, 1981), and some kinetic properties of the enzyme preparation, measured with a more sensitive, continuous assay method, are described in the present paper.

Studies of the metal-ion-dependency of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases have also yielded apparently conflicting results. The phenylalanine-sensitive isoenzyme from *E. coli* was found to be inhibited by EDTA and activated by Co^{2+} and Mn^{2+} (Staub & Dénes, 1969a,b; Simpson *et al.*, 1971; McCandliss & Herrmann, 1978; McCandliss *et al.*, 1978), yet Simpson & Davidson

(1976) found no such effects. The tyrosine-sensitive isoenzyme from *E. coli* was found to be inactivated by EDTA by Dusha & Dénes (1976), yet Schoner & Herrmann (1976) found that the enzyme was insensitive to EDTA. Both these enzymes were subsequently shown to be metalloenzymes containing Fe^{2+} (McCandliss & Herrmann, 1978). Similarly, the tyrosine-sensitive isoenzyme from *S. typhimurium* was found to be inactivated by EDTA and activated by Co^{2+} (Nagano & Zalkin, 1970), whereas De Leo *et al.* (1973) found no such effects, and the enzyme from *Bacillus subtilis* was found to be inactivated by EDTA and activated by Cd^{2+} and Co^{2+} (Huang *et al.*, 1974), yet these effects were not found by Jensen & Nester (1966). The effect of EDTA on the tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from *N. crassa* has not been examined before.

Materials and methods

Materials

ADP, fructose 6-phosphate, glycerol 3-phosphate dehydrogenase (EC 1.1.1.8), NAD^+ , NADPH, transaldolase (EC 2.2.1.2) and triose phosphate isomerase (EC 5.3.1.1) were obtained from Boehringer Corp., Lewes, Sussex, U.K. Indol-3-ylacetic acid, DL-indol-3-yl-lactic acid and indol-3-ylpropionic acid were obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. All other enzymes and reagents were obtained from sources described previously (Nimmo & Coggins, 1981).

Millipore filters (type GS, pore size $0.22\mu\text{m}$) were obtained from Millipore S.A., Molsheim, France.

Tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase was purified from *N. crassa* by the method of Nimmo & Coggins (1981).

Assay of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase activity

Enzyme activity was assayed by the method of Schoner & Herrmann (1976). The standard assay mixture (1 ml) contained 50 mM-Bistrispropane {1,3-bis[tris(hydroxymethyl)methylamino]propane}/HCl buffer, pH 7.4, $50\mu\text{M}$ -D-erythrose 4-phosphate, $50\mu\text{M}$ -phosphoenolpyruvate and enzyme. The reaction was initiated by the addition of enzyme (except where indicated), and the rate of decrease in absorbance at 232 nm was monitored at 25°C . For measurements at very low concentrations of substrate a full-scale deflexion of $0.05 A_{232}$ unit was used and all solutions were passed through Millipore filters to remove small particles. One unit is defined as the amount of enzyme that catalyses the conversion of $1\mu\text{mol}$ of substrate/min.

Determination of D-erythrose 4-phosphate

In solution D-erythrose 4-phosphate forms dimers

that are in slow equilibrium with monomers (Blackmore *et al.*, 1976). The concentrations of monomers and dimers were measured by two methods.

(a) D-Erythrose 4-phosphate was assayed by a modification of the method of Racker (1963). The assay mixture (1 ml) contained 70 mM-triethanolamine hydrochloride/KOH buffer, pH 7.6, 7 mM-EDTA, 0.24 mM-NADH, 0.5 mM-fructose 6-phosphate, 10 units of triose phosphate isomerase, 2 units of glycerol 3-phosphate dehydrogenase, 0.3 unit of transaldolase and sample. The reaction was initiated by the addition of transaldolase, and the oxidation of NADH was monitored at 340 nm at 25°C . Transaldolase does not appear to act on D-erythrose 4-phosphate dimers, and therefore the initial rapid oxidation of NADH provides a measure of the monomers. After this, the conversion of dimers into monomers becomes rate-limiting and the much slower oxidation, which takes several hours to reach completion, provides a measure of the dimers. More of each enzyme was added at the end of the incubation to check that all the D-erythrose 4-phosphate had been used up.

(b) D-Erythrose 4-phosphate was also assayed by using 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. The assay mixture (1 ml) contained 50 mM-Bistrispropane/HCl buffer, pH 7.4, 0.1 mM-phosphoenolpyruvate, 0.2 unit of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase and sample. The reaction was initiated by the addition of the synthase, and the conversion of phosphoenolpyruvate into 3-deoxy-D-arabino-heptulosonate 7-phosphate was monitored at 232 nm at 25°C . As in the assay described above, the D-erythrose 4-phosphate monomers are rapidly used up and can be distinguished from the dimers, which must slowly revert to monomers before 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase can act on them (see the Results section).

The two assay methods gave very similar results. Except where indicated, the concentrations of D-erythrose 4-phosphate quoted in the present paper refer to the monomers only.

Determination of phosphoenolpyruvate

Phosphoenolpyruvate was assayed by two methods.

(a) Phosphoenolpyruvate was assayed by the method of Czok & Eckert (1963).

(b) Phosphoenolpyruvate was also assayed by using 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. The assay mixture (1 ml) contained 50 mM-Bistrispropane/HCl buffer, pH 7.4, 0.1 mM-D-erythrose 4-phosphate, 0.05 unit of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase and sample. The reaction was initiated by the addition of the synthase, and the conversion of phosphoenol-

pyruvate into 3-deoxy-D-*arabino*-heptulosonate 7-phosphate was monitored at 232 nm at 25°C.

The two assay methods gave very similar results.

Determination of 3-deoxy-D-arabino-heptulosonate 7-phosphate

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate was assayed by using the first three activities of the *arom* multienzyme complex from *N. crassa*. The assay mixture (1 ml) contained 50 mM-Bistrispropane/HCl buffer, pH 7.4, 0.5 mM-NAD⁺, 0.1 mM-NADPH, 50 µg of *arom* multienzyme complex and sample. The reaction was initiated by the addition of NAD⁺, and the oxidation of NADPH was monitored at 340 nm at 25°C.

Determination of inorganic phosphate

P_i was determined by the method of Michelsen (1957).

Steady-state kinetics

Lines were fitted by the program LINFIT as described by Bevington (1969).

Results

Assay of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase activity

The assays were performed in Bistrispropane/HCl buffer as described by Schoner & Herrmann (1976). This buffer is particularly suitable for the assay of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase activity as it has a low absorbance at 232 nm, does not inhibit the enzyme and does not contain a primary amino group, which might react slowly with the aldehyde and ketone groups present in D-erythrose 4-phosphate and 3-deoxy-D-*arabino*-heptulosonate 7-phosphate. The enzyme was fairly insensitive to changes in the ionic strength of the assay mixture provided that the salt concentration was at least 20 mM; below this the enzyme was less active. NaCl or KCl at 100 mM had no effect on the activity of the enzyme. In the presence of 100 µM-phosphoenolpyruvate and 100 µM-D-erythrose 4-phosphate, the reaction velocity was proportional to the enzyme concentration over at least a 100-fold range of enzyme concentration (50 ng/ml–7 µg/ml).

Effect of substrate concentration

In solution, D-erythrose 4-phosphate forms dimers, which are in slow equilibrium with monomers (Blackmore *et al.*, 1976). The rate of inter-conversion of dimers and monomers was lower than the rates of conversion of D-erythrose 4-phosphate into 3-deoxy-D-*arabino*-heptulosonate 7-phosphate catalysed by the synthase in most of these experiments. Consequently care was taken to ensure that the measured rates of production of 3-deoxy-D-

arabino-heptulosonate 7-phosphate were due to the activity of the synthase and not to the rate of conversion of D-erythrose 4-phosphate dimers into monomers. At equilibrium the ratio of dimers to monomers was 2:1 in aqueous solution, and the ratio was unaffected by the components of the synthase assay mixtures. It was decided to determine whether D-erythrose 4-phosphate dimers were a substrate or an inhibitor of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase. An assay mixture (1 ml) was set up containing 100 µM-phosphoenolpyruvate and 0.03 unit of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase. Successive portions of D-erythrose 4-phosphate solution containing 10 nmol of monomers and 21 nmol of dimers were added to the assay mixture, and the conversion of phosphoenolpyruvate into 3-deoxy-D-*arabino*-heptulosonate 7-phosphate was monitored at 232 nm. After each addition the reaction was allowed to proceed until all the monomers had been used up and the rate-limiting step had become the conversion of dimers into monomers. Thus immediately after each addition the concentration of D-erythrose 4-phosphate monomers was the same (10 µM) but the concentration of dimers increased each time. The enzyme was stable during the experiment. It was found that the enzyme rate for a given concentration of D-erythrose 4-phosphate monomers was independent of the concentration of the dimers. On the other hand, the rate of reaction when all the monomers had been used up was directly proportional to the concentration of dimers, and this rate was not increased by adding more enzyme. This supports the idea that under these conditions the rate-limiting step was the non-enzymic conversion of D-erythrose 4-phosphate dimers into monomers. It therefore seems that D-erythrose 4-phosphate dimers are neither a substrate nor an inhibitor of the enzyme at the concentrations used in the following experiments, and their presence has been ignored. Since the two assay methods for D-erythrose 4-phosphate gave very similar results, it seems likely that transaldolase does not use D-erythrose 4-phosphate dimers as a substrate either.

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase gave rise to linear double-reciprocal plots of initial velocity against phosphoenolpyruvate concentration and D-erythrose 4-phosphate concentration (Fig. 1). The enzyme was inhibited by high concentrations of either substrate (not shown). In the double-reciprocal plot of velocity against D-erythrose 4-phosphate concentration at a series of fixed concentrations of phosphoenolpyruvate the lines appeared to intersect on the ordinate (results not shown). This pattern is indicative of a Rapid-Equilibrium Ordered mechanism (Cleland, 1970), in which phosphoenolpyruvate is the first substrate to

Fig. 1. Effect of substrate concentration on 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. Double-reciprocal plot of initial velocity (v) against [phosphoenolpyruvate] at a series of fixed concentrations of erythrose 4-phosphate: ○, $6.4 \mu\text{M}$; ▲, $8.0 \mu\text{M}$; □, $10 \mu\text{M}$; ●, $13 \mu\text{M}$; △, $16 \mu\text{M}$; ■, $22 \mu\text{M}$; ▽, $32 \mu\text{M}$. Inset: variations of the slopes (○) and intercepts (■) (expressed in arbitrary units) as a function of [erythrose 4-phosphate] ($[\text{Ery-4-P}]$).

bind with a K_s of $12 \mu\text{M}$ and D-erythrose 4-phosphate binds second with a K_m of $2.7 \mu\text{M}$ (Fig. 1).

Product-inhibition studies

These were conducted in order to determine the order of release of the products. All the double-reciprocal plots of initial rate against substrate concentration at a series of fixed concentrations of product were linear, as were the replots of the slopes and intercepts (Figs. 2 and 3). The results of these studies are summarized in Table 1. The patterns are consistent with the proposed Rapid-Equilibrium Ordered mechanism. 3-Deoxy-D-arabino-heptulosonate 7-phosphate appeared to be a competitive inhibitor with respect to phosphoenolpyruvate and D-erythrose 4-phosphate (Fig. 2), whereas P_i was a non-competitive inhibitor (Fig. 3), and this indicates that 3-deoxy-D-arabino-heptulosonate 7-phosphate is the last product to be released (Cleland, 1970). These patterns are also consistent with a Rapid-Equilibrium Random mechanism (Cleland, 1970) in which dead-end complexes are formed between enzyme, P_i and D-erythrose 4-phosphate and between enzyme, P_i and phosphoenolpyruvate. Binding studies might help to distinguish between these

Fig. 2. Inhibition of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase by 3-deoxy-D-arabino-heptulosonate 7-phosphate

(a) Double-reciprocal plot of initial velocity (v) against [phosphoenolpyruvate] at a series of fixed concentrations of 3-deoxy-D-arabino-heptulosonate 7-phosphate: ○, $0 \mu\text{M}$; ▲, $60 \mu\text{M}$; □, $120 \mu\text{M}$; ●, $180 \mu\text{M}$; △, $240 \mu\text{M}$. Assay mixtures contained $23 \mu\text{M}$ -erythrose 4-phosphate. (b) Double-reciprocal plot of initial velocity (v) against [erythrose 4-phosphate] at a series of fixed concentrations of 3-deoxy-D-arabino-heptulosonate 7-phosphate: ○, $0 \mu\text{M}$; ▲, $60 \mu\text{M}$; □, $120 \mu\text{M}$; ●, $240 \mu\text{M}$; △, $360 \mu\text{M}$. Assay mixtures contained $44 \mu\text{M}$ -phosphoenolpyruvate. Insets: variation of the slopes (arbitrary units) as a function of [3-deoxy-D-arabino-heptulosonate 7-phosphate] ($[\text{dHepA-7-P}]$).

mechanisms. However, in view of the results shown in Fig. 1, a Rapid-Equilibrium Ordered mechanism seems more likely.

Fig. 3. Inhibition of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase by P_i

(a) Double reciprocal plot of initial velocity (v) against [phosphoenolpyruvate] at a series of fixed concentrations of P_i : $\circ$, 0 mM; $\blacktriangle$, 2.5 mM; $\square$, 5.0 mM; $\bullet$, 7.5 mM; $\triangle$, 10.0 mM; $\blacksquare$, 12.5 mM. Assay mixtures contained $22\ \mu\text{M}$ -erythrose 4-phosphate. (b) Double-reciprocal plot of initial velocity (v) against [erythrose 4-phosphate] at a series of fixed concentrations of P_i : $\bullet$, 0 mM; $\triangle$, 5 mM; $\blacksquare$, 10 mM; $\circ$, 15 mM; $\blacktriangle$, 20 mM; $\square$, 25 mM. Assay mixtures contained $40\ \mu\text{M}$ -phosphoenolpyruvate. Insets: variations of the slopes ($\circ$) and intercepts ($\blacksquare$) (arbitrary units) as a function of $[P_i]$.

Stoichiometry, equilibrium and reversibility of the 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase reaction

To determine the stoichiometry of the reaction, 0.03 unit of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase/ml was incubated overnight at room temperature with 50 mM-Bistrispropane/HCl buffer, pH 7.4, 1.2 mM-phosphoenolpyruvate, 1.1 mM-D-erythrose 4-phosphate monomers and 2.2 mM-D-erythrose 4-phosphate dimers. After the incubation, 1.3 mM-3-deoxy-D-arabino-heptulosonate 7-phosphate and 1.1 mM- P_i had been formed and 0 mM-phosphoenolpyruvate, 0.7 mM-D-erythrose 4-phosphate monomers and 1.3 mM-D-erythrose 4-phosphate dimers remained. Therefore the enzyme converts one molecule of phosphoenolpyruvate and one molecule of D-erythrose 4-phosphate into one molecule of 3-deoxy-D-arabino-heptulosonate 7-phosphate and one molecule of P_i . When phosphoenolpyruvate and the synthase or D-erythrose 4-phosphate and the synthase were incubated under the same conditions, less than 5% of the phosphate was released from the substrate.

The equilibrium of the reaction appears to lie well in favour of 3-deoxy-D-arabino-heptulosonate 7-phosphate formation. No reverse reaction was observed even under conditions designed to displace the equilibrium of the reaction. Thus no reaction was observed when 0.03 unit of the synthase/ml was incubated with 0.7 mM-3-deoxy-D-arabino-heptulosonate 7-phosphate and 20 mM- P_i in the presence of the D-erythrose 4-phosphate assay mixture (a) or when the enzyme was incubated with 0.7 mM-3-deoxy-D-arabino-heptulosonate 7-phosphate and 2 mM- P_i in the presence of the D-erythrose 4-phosphate assay mixture (a) and the phosphoenolpyruvate assay mixture (a).

Inhibition by L-tryptophan

Since tryptophan has a primary amino group, it might react with D-erythrose 4-phosphate. To test this, the spectra of tryptophan and D-erythrose 4-phosphate were scanned separately and together in

Table 1. Inhibitor constants for the inhibition of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase by its products. Abbreviations used: dHepA-7-P, 3-deoxy-D-arabino-heptulosonate 7-phosphate; Ery-4-P, erythrose 4-phosphate; Pyr-P, phosphoenolpyruvate.

Variable product	Variable substrate	Concn. of fixed substrate	Inhibition pattern	K_{is}	K_{ii}
P_i	Ery-4-P	$40\ \mu\text{M}$ -Pyr-P	Non-competitive	11.5 mM	26 mM
P_i	Pyr-P	$20\ \mu\text{M}$ -Ery-4-P	Non-competitive	4.3 mM	33 mM
dHepA-7-P	Ery-4-P	$44\ \mu\text{M}$ -Pyr-P	Competitive	$85\ \mu\text{M}$	—
dHepA-7-P	Pyr-P	$23\ \mu\text{M}$ -Ery-4-P	Competitive	$40\ \mu\text{M}$	—

the range 230–282 nm, and no differences were observed. Furthermore, samples of D-erythrose 4-phosphate were assayed by using method (a) with and without a preincubation with tryptophan, and exactly the same progress curve for the conversion of D-erythrose 4-phosphate into sedoheptulose 7-phosphate was observed in each case. It therefore seems unlikely that there is any significant interaction between D-erythrose 4-phosphate and tryptophan during the assay of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase activity.

Double-reciprocal plots of initial rate against substrate concentration at a series of fixed concentrations of tryptophan were linear, and tryptophan appeared to be a non-competitive inhibitor with respect to phosphoenolpyruvate (Fig. 4a) and a competitive inhibitor with respect to D-erythrose 4-phosphate (Fig. 4b). The slope and intercept replots were upwardly curved in each case.

Tyrosine and phenylalanine did not inhibit the enzyme at concentrations of 1 mM (Nimmo & Coggins, 1981), and the tryptophan analogues DL-indol-3-yl-lactic acid, indol-3-ylpropionic acid and indol-3-ylacetic acid did not inhibit the enzyme at concentrations of 100 μ M.

Inactivation by EDTA

When EDTA was present in the standard 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase assay mixture, it was observed that the order of addition of the components of the assay mixture affected the enzyme rate. When the reaction was started by the addition of D-erythrose 4-phosphate or enzyme, a normal linear rate was observed, and this was unaffected by EDTA. However, when the reaction was started by the addition of phosphoenolpyruvate, a much lower rate was observed, which accelerated over the first few minutes and then became linear: the final rate was still lower than the normal rate described above. (In each case the assay was warmed for 5 min before the reaction was started.) Thus phosphoenolpyruvate can stabilize the synthase against inactivation by EDTA, and the inactivation can be partially but not completely reversed by phosphoenolpyruvate.

In further experiments the activity of the enzyme was measured after the initial recovery of activity described above. By using this procedure the inactivation by EDTA at 25°C was found to be first-order with respect to time. A plot of $\log(1/t_4)$ against $\log[\text{EDTA}]$ was linear with a slope of 1.0 (Fig. 5), indicating that the inactivation was first-order with respect to [EDTA]. There was no detectable inactivation of the synthase due to EDTA at 0°C. A number of reagents were tested for the ability to protect the synthase against inactivation by 0.1 mM-EDTA at 25°C. Phosphoenolpyruvate, 3-deoxy-D-arabino-heptulosonate 7-phosphate and

Fig. 4. Inhibition of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase by L-tryptophan

(a) Double-reciprocal plot of initial velocity (v) against [phosphoenolpyruvate] at a series of fixed concentrations of tryptophan: $\circ$, 0 μ M; $\blacktriangle$, 5 μ M; $\square$, 10 μ M; $\bullet$, 20 μ M; $\triangle$, 25 μ M; $\blacksquare$, 30 μ M. Assay mixtures contained 20 μ M-erythrose 4-phosphate. (b) Double-reciprocal plot of initial velocity (v) against [erythrose 4-phosphate] at a series of fixed concentrations of tryptophan: $\circ$, 0 μ M; $\blacktriangle$, 5 μ M; $\square$, 7 μ M; $\bullet$, 10 μ M; $\triangle$, 12 μ M; $\blacksquare$, 15 μ M; ∇ , 20 μ M. Assay mixture contained 40 μ M-phosphoenolpyruvate. Insets: variations of the slopes ($\circ$) and intercepts ($\blacksquare$) (arbitrary units) as a function of [tryptophan] ([Trp]).

several bivalent metal ions (Mn^{2+} , Co^{2+} , Zn^{2+} , Ca^{2+} and Mg^{2+}) protected the enzyme, whereas D-erythrose 4-phosphate (0.4 mM) and tryptophan (20 μ M) did not. P_i (50 mM) gave slight protection,

Fig. 5. Determination of the order of the inactivation of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase by EDTA with respect to [EDTA]

3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase (2 units/ml) was diluted 100-fold into 50 mM-Bistrispropane/HCl buffer, pH 7.4, containing various concentrations of EDTA and was incubated at 25°C. At intervals the 3-deoxy-D-arabino-heptulosonate synthase activity was measured by withdrawing samples and diluting them 10-fold into the standard assay mixture. Plots of log(synthase activity) against time were linear for the first 20 min after the addition of EDTA, and the half-time of inactivation (t_1) for each [EDTA] value was calculated from the slopes of these plots. These data were replotted as $\log(1/t_1)$ against $\log[EDTA]$, where [EDTA] is measured in μM and t_1 in minutes.

Fig. 6. Determination of the binding constant of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase for 3-deoxy-D-arabino-heptulosonate 7-phosphate

3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase (2 units/ml) was diluted 100-fold into 50 mM-Bistrispropane/HCl buffer, pH 7.4, containing 0.1 mM-EDTA and various concentrations of 3-deoxy-D-arabino-heptulosonate 7-phosphate (0–350 μM) and was incubated at 25°C. The rates of inactivation of the enzyme were measured as described in Fig. 5. From plots of $\log(\text{synthase activity})$ against time the pseudo-first-order rate constants for inactivation in the presence of $x \mu\text{M}$ -3-deoxy-D-arabino-heptulosonate 7-phosphate (v_x) and in the absence of 3-deoxy-D-arabino-heptulosonate 7-phosphate (v_0) were calculated in min^{-1} . These data were replotted as v_x/v_0 against $[1 - (v_x/v_0)]/[d\text{HepA-7-P}]$, and the slope is equal to the binding constant for 3-deoxy-D-arabino-heptulosonate 7-phosphate (dHepA-7-P), (Scrutton & Utter, 1965).

but no more than that afforded by a solution of KCl of equivalent ionic strength. From studies of the time courses of inactivation by 0.1 mM-EDTA in the presence of various concentrations of 3-deoxy-D-arabino-heptulosonate 7-phosphate and phosphoenolpyruvate, binding constants for these two ligands were calculated by the method of Scrutton & Utter (1965) (Figs. 6 and 7). Both plots were straight lines that did not pass through the origin, indicating that there is some inactivation of the enzyme by EDTA even in the presence of saturating concentrations of phosphoenolpyruvate or 3-deoxy-D-arabino-heptulosonate 7-phosphate. From the slope of the plot of v_x/v_0 against $[1 - (v_x/v_0)]/[d\text{HepA-7-P}]$ the binding constant for 3-deoxy-D-arabino-heptulosonate 7-phosphate (dHepA-7-P), was found to be 34 μM (Fig. 6), which compares well with the value of 40 μM obtained from product-inhibition studies (Table 1 and Fig. 2a). Similarly,

from the slope of the plot shown in Fig. 7 the binding constant for phosphoenolpyruvate was found to be 4.2 μM , which is somewhat lower than the value of 12 μM obtained from the kinetic studies shown in Fig. 1. The bivalent metal ions appeared to protect by binding to the EDTA rather than to the enzyme, since in each case the binding constant, measured by the same procedure as that used for 3-deoxy-D-arabino-heptulosonate 7-phosphate and phosphoenolpyruvate (Figs. 6 and 7), was found to be approximately half the concentration of EDTA present.

3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase was not activated by bivalent metal ions. At 1 mM, Mg^{2+} and Ca^{2+} had no effect, and Zn^{2+} , Co^{2+} and Mn^{2+} inhibited the enzyme. The tyrosine-sensitive and phenylalanine-sensitive isoenzymes from *E. coli* have been shown to contain

Fig. 7. Determination of the binding constant of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase for phosphoenolpyruvate

3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase (2 units/ml) was diluted 100-fold into 50 mM-Bistrispropane/HCl buffer, pH 7.4, containing 0.1 mM-EDTA and various concentrations of phosphoenolpyruvate (0–71 μ M). The rates of inactivation of the enzyme were measured as described in Fig. 5. These data were replotted as described in Fig. 6 to obtain the binding constant for phosphoenolpyruvate (Pyr-P).

Fe^{2+} , and they have a characteristic absorbance peak at 350 nm (McCandliss & Herrmann, 1978). The tryptophan-sensitive isoenzyme from *N. crassa* did not have an absorbance peak in this region. Over a long period of time, even in the absence of EDTA, the enzyme lost activity slowly, and Mg^{2+} and phosphoenolpyruvate were both able to stabilize the enzyme against this loss of activity.

Discussion

The effects of substrate concentration and products on the activity of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase were consistent with a Rapid-Equilibrium Ordered mechanism for the enzyme. This is in contrast with the Ping Pong mechanism proposed for this enzyme by Ip & Doy (1979), although in both cases it is proposed that phosphoenolpyruvate is the first substrate to bind and 3-deoxy-D-arabino-heptulosonate 7-phosphate is the last product to be released. The K_m values for D-erythrose 4-phosphate and phosphoenolpyruvate (130 μ M and 100 μ M respectively) measured by Ip & Doy (1979) are much higher than the values obtained from Fig. 1 (2.7 μ M for D-erythrose 4-phosphate and an 'apparent K_m ' of 0 μ M for phos-

phoenolpyruvate), and the patterns of inhibition by phosphate obtained by Ip & Doy (1979) are different from those shown in Fig. 3. The reasons for these discrepancies are unclear. Phosphoenolpyruvate and 3-deoxy-D-arabino-heptulosonate 7-phosphate protected the synthase against inhibition by EDTA (Figs. 6 and 7), whereas D-erythrose 4-phosphate and P_i did not, and this is consistent with the proposed ordered mechanism in which phosphoenolpyruvate and 3-deoxy-D-arabino-heptulosonate 7-phosphate can bind to the free enzyme whereas D-erythrose 4-phosphate and P_i cannot. This is the first 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase for which a Rapid-Equilibrium Ordered mechanism has been proposed. This mechanism is only a special case of the Sequential Ordered mechanism (Cleland, 1970), with the same order of addition of substrates and release of products, which has been proposed for several other 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases (see, e.g., Simpson & Davidson, 1976; De Leo *et al.*, 1973; Schoner & Herrmann, 1976; Sugimoto & Shiio, 1980; Dusha & Dènes, 1976).

Although there is no reason why all 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases should obey one type of mechanism, it seems clear that some of the disagreements about the kinetics of these enzymes have been caused by the discontinuous assay methods (see, e.g., Doy & Brown, 1965) favoured by many groups. The continuous assay method of Schoner & Herrmann (1976) is more suitable for kinetic studies. Several 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases have very low K_m values for phosphoenolpyruvate and D-erythrose 4-phosphate. Consequently the kinetics have to be studied at low concentrations of substrate, and it is important to ensure that the enzyme does not consume significant amounts of one substrate during the time taken to measure the initial rate. D-Erythrose 4-phosphate forms dimers in solution, as already discussed, and the chemical assay for D-erythrose 4-phosphate (Dische & Dische, 1958) does not distinguish between dimers and monomers, whereas the enzyme does, so that the concentration of this substrate available to the enzyme could be overestimated. If all the monomers were consumed during the measurement of the initial velocity, then the rate-determining step could become the conversion of D-erythrose 4-phosphate dimers into monomers. Buffers containing primary amino groups should be avoided, since they may react with D-erythrose 4-phosphate and 3-deoxy-D-arabino-heptulosonate 7-phosphate, and the use of phosphate as the assay buffer may cause problems, since it is a product of the enzyme.

The inhibition of the enzyme by tryptophan was not hyperbolic, and was competitive with respect to

D-erythrose 4-phosphate (Fig. 4b) and non-competitive with respect to phosphoenolpyruvate (Fig. 4a), in qualitative agreement with the results obtained by Ip & Doy (1979). The upwardly curving slope and intercept replots may indicate that tryptophan can bind to the enzyme more than once in the reaction sequence. Tryptophan did not protect the enzyme itself, nor prevent the protection by phosphoenolpyruvate against inactivation by EDTA, and this is consistent with tryptophan being a non-competitive inhibitor with respect to phosphoenolpyruvate (Fig. 4a).

The inactivation of the enzyme by EDTA suggests that the tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from *N. crassa* may be a metalloenzyme. The inactivation was not observed in the presence of phosphoenolpyruvate or at low temperatures, and is thus very similar to the inactivation of the phenylalanine-sensitive synthase from *E. coli* (Staub & Dénes, 1969a,b), which was likewise not observed at low temperatures (McCandliss & Herrmann, 1978) or in the presence of phosphoenolpyruvate (Simpson & Davidson, 1976). These observations could account for several of the discrepancies in the literature. Thus tyrosine-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from *S. typhimurium* was inactivated by EDTA in the absence of phosphoenolpyruvate (Nagano & Zalkin, 1970) but not in its presence (De Leo *et al.*, 1973), and the tyrosine-sensitive enzyme from *E. coli* was inactivated by EDTA at higher temperatures (Dusha & Dénes, 1976) but not in the cold (Schoner & Herrmann, 1976). Such considerations do not account for the observation of inactivation by EDTA of the *B. subtilis* enzyme by Huang *et al.* (1974) but not by Jensen & Nester (1966). There have been many reports of activation of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases by bivalent metal ions and of bivalent metal ions providing protection against and re-activation after inactivation by EDTA. The tryptophan-sensitive enzyme from *N. crassa* was protected by bivalent metal ions against inactivation by EDTA. Such observations do not indicate the nature of the metal ion present in the enzyme, however, since the phenylalanine-sensitive enzyme from *E. coli*, which contains Fe^{2+} , was not re-activated by Fe^{2+} after inactivation by EDTA (McCandliss & Herrmann, 1978) and was protected by several bivalent metal ions (Staub & Dénes, 1969a). It seems probable that many of the 3-deoxy-D-arabino-heptulosonate 7-phosphate synthases are metalloenzymes.

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