The purification of shikimate dehydrogenase from *Escherichia coli*

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A procedure was developed for the purification of shikimate dehydrogenase from *Escherichia coli*. Homogeneous enzyme with specific activity 1100 units/mg of protein was obtained in 21% overall yield. The subunit M_r estimated by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was 32 000. The native M_r , estimated by gel-permeation chromatography on a TSK G2000SW column, was also 32 000. *E. coli* shikimate dehydrogenase is therefore a monomeric NADP-linked dehydrogenase.

In micro-organisms and plants the biosynthesis of aromatic amino acids proceeds by way of the shikimate pathway (Haslam, 1974; Weiss & Edwards, 1980). The first seven steps on this pathway lead from erythrose 4-phosphate via shikimate to chorismate, which is the common precursor of all the aromatic amino acids (Gibson & Pittard, 1968) (see Scheme 1). One of the remarkable features of this pathway is the very different patterns of enzyme organization found in different species. It has been demonstrated that in *Neurospora crassa* five of the seven enzymes (those catalysing steps 2–6 in Scheme 1) occur as a multienzyme complex, which consists of two identical pentafunctional polypeptide chains of M_r 165 000 (Lumsden & Coggins, 1977, 1978; Gaertner & Cole, 1977; Smith & Coggins, 1983; Lambert *et al.*, 1985). In contrast, in *Escherichia coli* the corresponding enzymes are separable (Berlyn & Giles, 1969). Only three of these separable *E. coli* enzymes, 3-dehydroquinase synthase (Maitra & Sprinson, 1978; Frost *et al.*, 1984), 3-dehydroquinase (S. Chaudhuri & J. R. Coggins, unpublished work) and 5-enolpyruvylshikimate phosphate synthase (Lewendon & Coggins, 1983; Duncan *et al.*, 1984), have been purified to homogeneity. The other two, shikimate dehydrogenase (Yaniv & Gilvarg, 1955) and shikimate kinase (Ely & Pittard, 1979), have been purified to only a very limited extent.

It has been shown that limited proteolysis of the pentafunctional *Neurospora arom* enzyme complex results in the formation of a bifunctional polypeptide of M_r 68 000, which carries both 3-dehydroquinase and shikimate dehydrogenase activities (Smith & Coggins, 1983). We wished to compare this bifunctional fragment of the *arom* polypeptide

with the two corresponding *E. coli* enzymes. In order to do so we have devised a method to purify shikimate dehydrogenase from *E. coli*. In the present paper we describe the purification procedure and report some of the properties of the purified enzyme.

Materials and methods

Materials

All the proteins and enzymes were obtained from either Boehringer Corp., Lewes, East Sussex, U.K., or Sigma Chemical Co., Poole, Dorset, U.K. DEAE-Sephacel, Sephacryl S-200 (superfine grade) and ADP-Sepharose were from Pharmacia, Milton Keynes, Bucks., U.K. The other reagents were obtained from BDH Chemicals, Poole, Dorset, U.K., Aldrich Chemical Co., Gillingham, Dorset, U.K., or Koch-Light, Haverhill, Suffolk, U.K.

Purification of shikimate dehydrogenase

All steps were carried out 4°C unless otherwise stated.

Step 1: extraction and centrifugation. A 20 g batch of *E. coli* (strain A.T.C.C. 14948) cells was suspended in 10 ml of 100 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-EDTA, 0.4 mM-dithiothreitol and 1.2 mM-phenylmethanesulphonyl fluoride and broken by two passages through a French pressure cell. The extract was diluted with the above buffer to 80 ml, deoxyribonuclease I (0.5 mg) was added and after 1 h stirring the resulting suspension was centrifuged at 28 000 g for 30 min. The supernatant was the crude extract from which the enzyme was purified.

Scheme 1. Reactions of the early common pathway of aromatic amino acid biosynthesis

The numbers on the scheme refer to the enzymes of the pathway: 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (EC 4.1.2.15); 2, 3-dehydroquinate synthase (EC 4.6.1.3); 3, 3-dehydroquinase (EC 4.2.1.10); 4, shikimate dehydrogenase (EC 1.1.1.25); 5, shikimate kinase (EC 2.7.1.71); 6, 5-enolpyruvoylshikimate 3-phosphate synthase (EC 2.5.1.19; alternative name 3-phosphoshikimate 1-carboxyvinyltransferase); 7, chorismate synthase (EC 4.6.1.4).

Step 2: fractionation with $(NH_4)_2SO_4$. The crude extract was made 1M in benzamidine and adjusted to 30% saturation with solid $(NH_4)_2SO_4$ (176g/l). After the mixture had been stirred for 20min, the precipitate was removed by centrifugation at 28000g for 30min. The supernatant was adjusted to 55% saturation with solid $(NH_4)_2SO_4$ (338g/l) and stirred for 20min. The precipitated protein was collected by centrifugation at 17000g for 30min, redissolved in 50mM-Tris/HCl buffer, pH 7.5, containing 0.4mM-dithiothreitol and 1.2mM-phenylmethanesulphonyl fluoride (buffer A), and dialysed overnight against 2 x 1 litre of 50mM-Tris/HCl buffer, pH 7.5, containing 0.4mM-dithiothreitol, 1.2mM-phenylmethanesulphonyl fluoride and 50mM-KCl (buffer B).

Step 3: DEAE-Sephacel chromatography. The dialysed protein was applied to a DEAE-Sephacel column (14cm x 2.1cm) that had been pre-equilibrated with buffer B. The column was washed with

the buffer B until the A_{280} was below 0.2 (flow rate 60ml/h; 10ml fractions). The protein was eluted with a 700ml linear gradient of KCl (50–350mM) in buffer B (flow rate 36ml/h; 6ml fractions). The fractions containing enzyme activity were pooled and dialysed overnight against 1 litre of 25mM-Tris/HCl buffer, pH 7.5, containing 0.4mM-dithiothreitol and 1.2mM-phenylmethanesulphonyl fluoride (buffer C). The protein was concentrated by adsorption on a 2ml (bed volume) DEAE-Sephacel column and elution with buffer C containing 1M-KCl. The concentrated enzyme was dialysed against 50mM-Tris/HCl buffer, pH 7.5, containing 20% (v/v) glycerol and 0.4mM-dithiothreitol.

Step 4: Sephacryl S200 chromatography. The concentrated enzyme solution was applied to a Sephacryl S-200 (superfine grade) column (85cm x 2.1cm) that had been equilibrated with 50mM-Tris/HCl buffer, pH 7.5, containing

500mM-KCl and 0.4mM-dithiothreitol. The enzyme was eluted with the same buffer (flow rate 4ml/h; 1 ml fractions). Fractions containing shikimate dehydrogenase activity were pooled and dialysed against 1 litre of buffer C.

Step 5: ADP-Sepharose chromatography. The dialysed enzyme was applied to an ADP-Sepharose column (5ml bed volume) that had been pre-equilibrated with buffer C. The column was washed with buffer C until the A_{280} of the eluate was zero (flow rate 8ml/h; 4ml fractions). Then a solution of 1mM-NADP⁺ in buffer C (flow rate 1ml/h; 1ml fractions) was used to elute the shikimate dehydrogenase activity. Fractions containing the activity were pooled and dialysed overnight against 1 litre of buffer A. The enzyme was further dialysed against 500ml of 50mM-Tris/HCl buffer, pH 7.5, containing 50% (v/v) glycerol, 1mM-benzamidine and 0.4mM-dithiothreitol (buffer D). At this stage the enzyme was routinely stored at -20°C.

Step 6: chromatography on Mono Q. This step was carried out at room temperature with a Pharmacia FPLC system. The enzyme from the previous step was diluted with 20mM-Tris/HCl buffer, pH 7.5, containing 20mM-KCl and 0.4mM-dithiothreitol and applied to a Mono Q column. The enzyme was eluted with a linear gradient of 20–300mM-KCl in the above buffer (flow rate 1 ml/min; 0.5ml fractions). The fractions containing shikimate dehydrogenase activity were pooled and dialysed overnight against buffer D before long term storage at -20°C.

Assay of shikimate dehydrogenase

The enzyme was assayed (in the reverse direction) at 25°C by monitoring the reduction of NADP⁺ at 340nm ($\epsilon = 6.18 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The assay mixture (total volume 1 ml) contained (final concentrations) 100mM-Na₂CO₃, pH 10.6, 4mM-shikimic acid and 2mM-NADP⁺. One unit of enzyme activity is defined as the amount of enzyme that catalyses the conversion of 1 μmol of substrate/min.

Determination of protein

Protein was determined by the method of Bradford (1976), with bovine serum albumin as standard.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed with a 3.5% stacking gel and a 12.5% running gel, by the method of Laemmli (1970). Gels were stained for protein with AgNO₃ (Eschenbruch & Burk, 1982) or with Coomassie Brilliant Blue as previously described (Lumsden & Coggins, 1977).

For determination of subunit M_r , a calibration line was constructed with the following marker proteins (numbers refer to Fig. 5): 1, bovine serum albumin (68000); 2, ovalbumin (43000); rabbit muscle fructose biphosphate aldolase (40000); 4, pig lactate dehydrogenase (36000); 5, bovine carbonic anhydrase (29000); 6, bovine chymotrypsinogen (25700); 7, soya-bean trypsin inhibitor (22000); 8, sperm-whale myoglobin (17200); 9, hen egg-white lysozyme (14300) (Weber & Osborn, 1969).

Simple polyacrylamide-gel electrophoresis was carried out as described previously (Lumsden & Coggins, 1977), except that 8.5% slab gels were used instead of tube gels. Gels were stained for protein or for shikimate dehydrogenase activity as described previously (Lumsden & Coggins, 1977).

Determination of M_r by gel filtration

The native M_r was estimated by gel-permeation chromatography at room temperature on a TSK G2000SW column (60cm $\times$ 0.75cm) (LKB, South Croydon, U.K.). The column was eluted with 0.067M-potassium phosphate buffer, pH 6.8 (flow rate 0.5ml/min), as described by Mousdale & Coggins (1984). The eluate was monitored at 215nm and the column was calibrated with the following proteins: bovine serum albumin (M_r 68000), chicken ovalbumin (M_r 45000), bovine erythrocyte carbonic anhydrase (M_r 29000) and sperm-whale myoglobin (M_r 17200) (Weber & Osborn, 1969).

Table 1. Purification scheme for *E. coli* shikimate dehydrogenase

The results presented are for a typical purification starting from 20g of *E. coli* cells (see the Materials and methods section).

Step	Volume (ml)	Concn. of protein (mg/ml)	Total protein (mg)	Activity (units/ml)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
1: Crude extract	68	18	1220	0.97	66	0.054	1	100
2: 30–55%-satn.(NH ₄) ₂ SO ₄	39	13.4	523	1.5	59	0.11	2	89
3: DEAE-Sepharcel	40	1.6	64	1.3	50	0.78	14	76
4: Sepharyl S-200	15	0.19	2.9	2.0	30	10	200	45
5: ADP-Sepharose	7.2	0.009	0.065	4.0	29	450	8900	45
6: Mono Q	0.46	0.029	0.013	31	14	1100	20000	21

Fig. 1. Chromatography of *E. coli* shikimate dehydrogenase on DEAE-Sephacel (step 3 of purification scheme). Enzyme from step 2 (59 units in 39 ml) was loaded on to a DEAE-Sephacel column (14 cm $\times$ 2.1 cm) in buffer B as described in the Materials and methods section. The column was eluted with a 700 ml linear gradient of KCl (50–350 mM) in buffer A. $\circ$, Protein (A_{280}); $\bullet$, shikimate dehydrogenase activity (units/ml); ----, conductivity (mS).

Results and discussion

The purification of *E. coli* shikimate dehydrogenase is summarized in Table 1. To minimize the risk of proteolysis during the purification procedure, either phenylmethanesulphonyl fluoride or benzamidine was included in all buffers (Lumsden & Coggins, 1977). After $(\text{NH}_4)_2\text{SO}_4$ fractionation the shikimate dehydrogenase activity was found in the 30–55% saturation fraction. The enzyme was eventually purified 10000-fold and in 24% yield from this fraction.

To obtain electrophoretically homogeneous enzyme, four chromatographic steps were required. The first step was ion-exchange chromatography on DEAE-Sephacel (Fig. 1), and the second was gel filtration on Sephacryl S200 (Fig. 2). At this stage the enzyme had been purified 200-fold, but polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate showed that there were still very many different polypeptide chains present (gel not shown).

The next step in the purification was affinity chromatography on ADP-Sepharose (Fig. 3). Shikimate dehydrogenase activity was eluted from the column with 1 mM-NADP⁺ in a single narrow band that clearly corresponded to a protein peak (Fig. 3). This step resulted in a 45-fold purification, and the recovery was 97%.

The final purification step was chromatography on a Mono Q column (Fig. 4). The purified enzyme showed a single protein band after polyacrylamide-gel electrophoresis in the presence of sodium

Fig. 2. Chromatography of *E. coli* shikimate dehydrogenase on Sephacryl S200 (step 4 of purification scheme). Enzyme from step 3 (50 units in 40 ml) was concentrated and applied to a Sephacryl S200 column (85 cm $\times$ 2.1 cm) and eluted as described in the Materials and methods section. $\circ$, Protein (mg/ml); $\bullet$, shikimate dehydrogenase activity (units/ml);

dodecyl sulphate (Fig. 5, panel I, track A), as well as a single protein band after simple polyacrylamide-gel electrophoresis (Fig. 5, panel II, track B). The R_F of this latter band corresponded exactly to the R_F of the single band of shikimate dehydrogenase activity observed when a simple polyacrylamide gel of the purified enzyme was

Fig. 3. Chromatography of *E. coli* shikimate dehydrogenase on ADP-Sepharose (step 5 of purification scheme). Enzyme from step 4 (30 units in 15 ml) was applied to an ADP-Sepharose column (bed volume 5 ml) in buffer C as described in the Materials and methods section. Shikimate dehydrogenase activity was eluted with 1 mM-NADP⁺ in buffer C. ○, Protein (mg/ml); ●, shikimate dehydrogenase activity (units/ml); ▲, specific activity (units/mg)

stained for enzyme activity (gel not shown). The specific activity of the purified enzyme was 1100 units/mg of protein.

The overall yield of the purification procedure was 21%. The enzyme was stable in crude extracts,

Fig. 4. Chromatography of *E. coli* shikimate dehydrogenase on Mono Q (step 6 of purification scheme). Enzyme from step 5 (29 units in 7 ml) was applied to a Mono Q column (bed volume 5 ml) in 20 mM-Tris/HCl buffer, pH 7.5, as described in the Materials and methods sections. Shikimate dehydrogenase activity was eluted with a 20–300 mM-KCl gradient. —, A_{280} ; ●, shikimate dehydrogenase activity (units/ml); ----, gradient trace from FPLC apparatus.

and after purification it was stable during prolonged storage (6 months) in buffer containing 50% glycerol. The low yield (48%) obtained from the Mono Q ion-exchange column was almost certainly due to the very low protein concentration at this final stage in the purification procedure and to the operation of the FPLC equipment at room temperature. In contrast, the earlier ion-exchange step on DEAE-Sepharose gave a good yield (85%).

The subunit M_r of *E. coli* shikimate dehydrogenase was estimated to be 32000 by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The native M_r , estimated by gel filtration on a TSK G2000SW column was 32000. These data indicate that *E. coli* shikimate dehydrogenase is unusual in being a monomeric enzyme. No other monofunctional, biosynthetic, NADP-linked shikimate dehydrogenase has been purified, and so it is not known whether this is a general feature of bacterial shikimate dehydrogenases. We are only aware of two other examples of monomeric NAD(P)-linked dehydrogenases. NADP-

linked dihydrofolate reductase from a variety of species is monomeric (Volz *et al.*, 1982). Also, an inducible catabolic NAD-linked enzyme, showing both quinate dehydrogenase and shikimate dehydrogenase activities, which has been purified

Fig. 5. Evidence that the purified shikimate dehydrogenase is homogeneous

Panel I shows two tracks from a 12.5%-polyacrylamide gel run in the presence of sodium dodecyl sulphate and stained by the silver method. Track A, 0.2 μ g of *E. coli* shikimate dehydrogenase eluted from the Mono Q column; track B, 0.5 μ g of purified *E. coli* 3-dehydroquinase (S. Chaudhuri & J. R. Coggins, unpublished work). The numbers on the right-hand side refer to the positions of marker proteins used for M_r calibration (for details see the Materials and methods section). Panel II shows a simple 8.5%-polyacrylamide slab gel that was stained for protein with Coomassie Blue. Track, A, 0.3 μ g of *E. coli* 3-dehydroquinase; track B, 0.15 μ g of *E. coli* shikimate dehydrogenase eluted from the Mono Q column.

from *N. crassa*, is monomeric, with an M_r of 41 000 (Lopez Barea & Giles, 1978). It will be interesting to see whether this enzyme is structurally related to *E. coli* shikimate dehydrogenase.

The fact that *E. coli* shikimate dehydrogenase is a simple monomeric protein contrasts with the situation found in *Neurospora* (Lumsden & Coggins, 1977, 1978; Gaertner & Cole, 1977) and *Saccharomyces* (Larimer *et al.*, 1983), where this enzyme is known to be part of the pentafunctional *arom* polypeptide chain. The *Neurospora arom* polypeptide is encoded by a single gene, whereas the five corresponding *E. coli* enzymes are each encoded by separate genes. The five *E. coli* genes are widely scattered (Pittard & Wallace, 1966), and the individual enzyme activities can be separated on sucrose density gradients (Berlyn & Giles, 1969). In plants it has generally been found impossible to separate shikimate dehydrogenase and 3-dehydroquinase (Boudet & Lecussan, 1974), and the two enzymes are believed to occur as a multienzyme complex. In the moss *Physcomitella patens* the multienzyme complex has been purified and the two enzymes were shown to occur on a single bifunctional polypeptide chain (Polley, 1978). In *Phaseolus mungo* seedlings (Koshiba, 1978) and *Pisum sativum* shoots (M. S. Campbell & J. R. Coggins, unpublished work) the multienzyme complex has been purified over 5000-fold, and both the enzymes co-purify in constant activity ratio, although the subunit structure is not known.

In *E. coli* shikimate dehydrogenase does not co-purify with 3-dehydroquinase. In crude extracts the two enzymes can be separated by $(\text{NH}_4)_2\text{SO}_4$ fractionation or gel filtration. It has been established that *E. coli* 3-dehydroquinase is a simple dimeric protein with a subunit M_r of 29 000 (S. Chaudhuri & J. R. Coggins, unpublished work). The subunit M_r values of *E. coli* shikimate dehydrogenase and 3-dehydroquinase differ only slightly, so that the purified enzymes are only just resolved on polyacrylamide-gel electrophoresis in the presence of the sodium dodecyl sulphate (Fig. 5, panel I). The two enzymes are clearly resolved on simple polyacrylamide-gel electrophoresis (Fig. 5, panel II).

The bifunctional fragment of the *Neurospora*

Table 2. Subunit structure of some of the pre-chorismate-pathway enzymes of *E. coli* and *N. crassa*
The pathway step numbers refer to Scheme 1.

Pathway step	<i>E. coli</i> subunit M_r	<i>N. crassa</i> subunit M_r	Reference for <i>E. coli</i> M_r
2	40 000	165 000	Frost <i>et al.</i> (1984)
3	29 000		S. Chaudhuri & J. R. Coggins (unpublished work)
4	32 000		Present paper
5	20 000		Ely & Pittard (1979)
6	46 000		Duncan <i>et al.</i> (1984)

arom multifunctional polypeptide, which carries the shikimate dehydrogenase and 3-dehydroquinase activities, has an M_r of 68000 (Smith & Coggins, 1983). This fragment can be further trimmed by treatment with trypsin to give a polypeptide of M_r 63000 that still carries both activities (Boocock, 1983). These data suggest that the *Neurospora* bifunctional domain may have arisen from the fusion of two genes like those found in *E. coli*. Gene fusions leading to multifunctional polypeptide chains have been reported for other enzymes involved in amino acid biosynthesis, for example for *E. coli* anthranilate synthase-anthranilate-5-phosphoribosylpyrophosphate phosphoribosyl-transferase (Miozzari & Yanofsky, 1979) and for yeast tryptophan synthase (Zalkin & Yanofsky, 1982). The gene-fusion hypothesis is attractive as a model for the origin of the entire *Neurospora arom* polypeptide, since the subunit M_r values of the five corresponding *E. coli* polypeptides add up to 167000 (see Table 2) and the *arom* subunit M_r is 165000 (Lumsden & Coggins, 1977). Sequence comparisons between the *arom* polypeptide and its *E. coli* counterparts will be necessary to provide evidence of such gene fusions.

Although the *Physcomitrella* polypeptide, of M_r 48000, appears to be too small to be simply related to the two *E. coli* polypeptides, it should be noted that one 3-dehydroquinase with a smaller subunit M_r has been characterized. This is the inducible catabolic 3-dehydroquinase of *N. crassa* (Hautala *et al.*, 1975), which is a dodecameric enzyme with a subunit M_r of 20000 (Hawkins *et al.*, 1982; Coggins & Chaudhuri, 1982). The minimum functional domain for 3-dehydroquinase may therefore be significantly smaller than that found in *E. coli*, and the *Physcomitrella* polypeptide may also be structurally related to the separable forms of 3-dehydroquinase and shikimate dehydrogenase.

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