Purification and characterization of 3-dehydroquinase from *Escherichia coli*

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A procedure has been developed for the purification of 3-dehydroquinase from *Escherichia coli*. Homogeneous enzyme with specific activity 163 units/mg of protein was obtained in 19% overall yield. The subunit M_r estimated from polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was 29000. The native M_r , estimated by gel permeation chromatography on Sephacryl S-200 (superfine) and on TSK G3000SW, was in the range 52000–58000, indicating that the enzyme is dimeric. The catalytic properties of the enzyme have been determined and shown to be very similar to those of the biosynthetic 3-dehydroquinase component of the *arom* multifunctional enzyme of *Neurospora crassa*.

INTRODUCTION

The chemical steps making up the early common pathway (the pre-chorismate pathway; see Scheme 1) for the biosynthesis of the aromatic amino acids in micro-organisms and plants were established more than 20 years ago (see reviews by Umbarger & Davis, 1962; Gibson & Pittard, 1968). One of the remarkable features

of this pathway is the very different patterns of enzyme organization found in different species. 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 165000 (Lumsden & Coggins, 1977, 1978; Gaertner & Cole, 1977; Smith & Coggins, 1983; Lambert *et al.*, 1985; Coggins *et al.*, 1985). In contrast,

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: (1) 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthase (EC 4.1.2.15), (2) 3-dehydroquinase 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).

in *Escherichia coli* the corresponding enzymes are separable (Berlyn & Giles, 1969; Chaudhuri & Coggins, 1985; Coggins *et al.*, 1985). Four of these separable *E. coli* enzymes, 3-dehydroquinase (Frost *et al.*, 1984), shikimate dehydrogenase (Chaudhuri & Coggins, 1985), shikimate kinase (Millar *et al.*, 1986) and 5-enolpyruvylshikimate phosphate synthase (Lewendon & Coggins, 1983) have been purified to homogeneity. However, the remaining enzyme, 3-dehydroquinase, has only been purified to a very limited extent from *E. coli* (Mitsuhashi & Davis, 1954).

Here we report the first purification to homogeneity of a monofunctional, biosynthetic 3-dehydroquinase and a comparison of its enzymic properties with those of the 3-dehydroquinase component of the *arom* penta-functional enzyme of *N. crassa*.

MATERIALS AND METHODS

Reagents

All the reagents and purified proteins and enzymes except those specified below were from BDH Chemicals, Boehringer Corp., Sigma Chemical Co. or Aldrich Chem. Co. DEAE-Sephacel and Sephacryl S-200 (superfine grade) were obtained from Pharmacia, and Bio-Gel HTP (hydroxyapatite) from Bio-Rad Laboratories.

Ammonium 3-dehydroquininate was prepared by the method of Grewe & Haendler (1968).

Purification of 3-dehydroquinase

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

Step 1: extraction and centrifugation. *E. coli* (strain A.T.C.C. 14948) cells (20g) were suspended in 10 ml of 100 mM-Tris/HCl, buffer, pH 7.5, containing 1 mM-EDTA, 1.2 mM-phenylmethanesulphonyl fluoride and 0.4 mM-dithiothreitol. The cells were broken by two passages through a French pressure cell and the extract was diluted with the above buffer to 80 ml. Deoxyribonuclease I (0.5 mg) was added and the extract stirred for 1 h and then centrifuged at 26000 *g* for 30 min to give a clear supernatant (the crude extract).

Step 2: fractionation with $(\text{NH}_4)_2\text{SO}_4$. The crude extract was made 1 mM in benzamidine and powdered $(\text{NH}_4)_2\text{SO}_4$ was slowly added with stirring to give a final concentration of 176 g/l (30% saturation). The solution was stirred for 20 min and then centrifuged as before. More $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to give a final concentration of 338 g/l (55% saturation), and the precipitate obtained after centrifugation at 17000 *g* for 30 min was retained and stored in buffer containing glycerol (50%, v/v) at -20 °C as it proved to be a useful source of shikimate dehydrogenase. More $(\text{NH}_4)_2\text{SO}_4$ was added to give a final concentration of 479 g/l (75% saturation) and the resulting precipitate was collected by centrifugation for 30 min at 26000 *g*. This precipitate was dissolved in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.4 mM-dithiothreitol, 1.2 mM-phenylmethanesulphonyl fluoride and dialysed overnight against 2 × 1000 ml of 50 mM-Tris/HCl buffer, pH 7.5, containing 0.4 mM-dithiothreitol, 1.2 mM-phenylmethanesulphonyl fluoride and 50 mM-KCl (buffer A).

Step 3: DEAE-Sephacel chromatography. The dialysed enzyme was loaded onto a column of DEAE-Sephacel (14 cm × 2.1 cm) which had been pre-equilibrated with buffer A (flow rate 60 ml/h; 10 ml fractions) until the A_{280} of the eluate was less than 0.1. The enzyme was then eluted with a linear gradient (700 ml) of 50–250 mM-KCl in buffer A (flow rate 36 ml/h; 6 ml fractions). The fractions containing 3-dehydroquinase activity were pooled and dialysed overnight against 1 litre of 25 mM-Tris/HCl buffer, pH 7.5, containing 0.4 mM-dithiothreitol and 1.2 mM-phenylmethanesulphonyl fluoride (buffer B). The enzyme was concentrated by binding it to a 2 ml (bed volume) DEAE-Sephacel column and eluted with buffer B containing 1 M-KCl. The concentrated enzyme was dialysed overnight against 2 × 1 litre of 60 mM-potassium phosphate buffer, pH 5.85, containing 0.4 mM-dithiothreitol and 1.2 mM-phenylmethanesulphonyl fluoride (buffer C).

Step 4: hydroxyapatite chromatography. The dialysed enzyme was loaded onto a column (bed volume 10 ml) of hydroxyapatite, previously equilibrated with buffer C (flow rate 10 ml/h, 2.5 ml fractions). The enzyme was eluted with a linear gradient (400 ml) of 60–300 mM-potassium phosphate buffer, pH 5.85, containing 0.4 mM-dithiothreitol and 1.2 mM-phenylmethanesulphonyl fluoride (flow rate 20 ml/h, 5 ml fractions). The fractions containing 3-dehydroquinase activity were pooled and dialysed overnight against 1 litre of buffer B. The enzyme was concentrated as previously described using DEAE-Sephacel and then dialysed for 6 h against 50 mM-Tris/HCl buffer, pH 7.5, containing 0.4 mM-dithiothreitol and 20% (v/v) glycerol.

Step 5: Sephacryl S-200 (superfine grade) chromatography. The concentrated enzyme was applied to a column (85 cm × 2.1 cm) of Sephacryl S-200 (superfine grade), pre-equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 0.4 mM-dithiothreitol and 500 mM-KCl. The enzyme was eluted with the same buffer (flow rate 6 ml/h; 1.5 ml fractions). Fractions containing 3-dehydroquinase activity were pooled. The pooled fractions were either concentrated using a DEAE-Sephacel column and then dialysed, or they were dialysed against 50 mM-Tris/HCl buffer, pH 7.5, containing 0.4 mM-dithiothreitol, 1 mM-benzamidine and 50% (v/v) glycerol. 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 using a Pharmacia FPLC system. The pool containing 3-dehydroquinase activity from the previous step was diluted to 5 ml with 20 mM-Tris/HCl buffer, pH 7.5, containing 20 mM-NaCl and 0.4 mM-dithiothreitol. This was applied to a Mono Q column and eluted with a three step linear gradient of 20–300 mM-NaCl in the above buffer (flow rate 1 ml/min; 0.25 ml fraction). Fractions containing 3-dehydroquinase activity were pooled and dialysed against 50 mM-Tris buffer, pH 7.5, containing 0.4 mM-dithiothreitol, 1 mM-benzamidine and 50% (v/v) glycerol for long term storage at -20 °C.

Assay of 3-dehydroquinase activity

To follow the purification the enzyme was assayed at 25 °C in 100 mM-potassium phosphate buffer, pH 7.0,

containing 0.33 mM-ammonium dehydroquinolate. Formation of 3-dehydroshikimate was monitored at 234 nm in a total volume of 1 ml ($\epsilon = 1.2 \times 10^4$ litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$). Enzyme activity during some of the other experiments was measured in 100 mM-potassium phosphate buffer, pH 7.5, at 25 °C as specified in the text.

One unit of enzyme activity is defined as the amount catalysing the conversion of 1 μ mol 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 SDS was performed according to the method of Laemmli (1970) with 3.5% stacking gel and 12.5% running gel. Gels were stained for protein with silver nitrate (Eschenbruch & Bürk, 1982).

Determination of subunit M_r by SDS/polyacrylamide-gel electrophoresis

A calibration line was constructed using the following marker proteins (numbers refer to Fig. 2): 1, bovine serum albumin (M_r 68 000); 2, ovalbumin (M_r 43 000); 3, rabbit muscle aldolase (M_r 40 000); 4, porcine lactate dehydrogenase (M_r 36 000); 5, bovine carbonic anhydrase (M_r 29 000); 6, bovine chymotrypsinogen (M_r 25 700); 7, soya bean trypsin inhibitor (M_r 22 000); 8, sperm whale myoglobin (M_r 17 200); 9, hen egg white lysosyme (M_r 14 300) (Weber & Osborn, 1969).

Determination of native M_r by gel filtration

The native M_r of 3-dehydroquinase was estimated by gel filtration on a Sephacryl S-200 (superfine grade) column (85 cm $\times$ 2.1 cm) and also by h.p.l.c. on a TSK G3000SW column (60 cm $\times$ 0.75 cm) using the equipment described previously (Mousdale & Coggins, 1984). The columns were developed with 50 mM-Tris/HCl buffer, pH 7.5, and 0.67 mM-potassium phosphate buffer, pH 6.8, respectively in the presence of 0.4 mM-dithiothreitol. The following proteins were used for calibration of the columns: horse heart cytochrome *c* (M_r 12 300; Margoliash *et al.*, 1961); myoglobin (M_r 17 200), chicken ovalbumin (M_r 43 000), bovine serum albumin (M_r 68 000; Weber & Osborn, 1969); hexokinase (M_r 102 000;

Lazarus *et al.*, 1968), pyruvate kinase (M_r 237 000; Steinmetz & Deal, 1966).

Inactivation with 3-dehydroquinolate and NaBH $_4$

Samples (200 μ l) of the two 3-dehydroquinases (10 μ M) in 50 mM-potassium phosphate buffer, pH 7.5, containing 0.4 mM-dithiothreitol were completely inactivated by adding ammonium dehydroquinolate to a final concentration of 1 mM followed by a total of 0.05 ml of freshly prepared 1 M-NaBH $_4$ in 50 mM-NaOH added in 10 μ l portions to minimize gas evolution. Control experiments carried out in the same manner but without the addition of 3-dehydroquinolate resulted in no detectable loss of enzyme activity.

Inactivation with diethylpyrocarbonate

Samples of the two 3-dehydroquinases were incubated in 50 mM-potassium phosphate buffer, pH 6.1, at 20 °C in the presence of diethylpyrocarbonate (final concentration in the range 0.1–4.0 mM). Aliquots were removed at intervals over a period of 20 min, quenched with 2 vol. of imidazole buffer (100 mM, pH 7.0) and assayed for 3-dehydroquinase activity at pH 7.0 and 25 °C.

Determination of kinetic parameters

K_m values were determined from double reciprocal plots and K_i values from secondary plots of apparent K_m values against inhibitor concentration (five inhibitor concentrations were used); lines were fitted by the programme LINFIT as described by Bevington (1969) and each experimental point was measured in triplicate.

RESULTS

Purification and subunit structure

The purification of *E. coli* 3-dehydroquinase is summarized in Table 1. After (NH $_4$) $_2$ SO $_4$ fractionation the 3-dehydroquinase activity was found in the 55–75%-saturation fraction. The enzyme was eventually purified 4060-fold and in 19% yield from this fraction.

To obtain electrophoretically homogeneous enzyme, four chromatographic steps were required. The first three steps involved ion-exchange chromatography on DEAE-Sephacel, ion-exchange chromatography on hydroxyapatite and gel filtration on Sephacryl S-200 (Fig. 1).

Table 1. Purification scheme for *E. coli* 3-dehydroquinase

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

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	22	1496	0.9	61.2	0.04	1	100
2. 55–75% satn. (NH $_4$) $_2$ SO $_4$	19.5	19.4	378	2.5	48.7	0.129	3.2	80
3. DEAE-Sephacel	52	0.33	17.2	0.8	41.6	2.42	60	68
4. Hydroxyapatite	43	0.054	2.3	0.92	40	17	426	65
5. Sephacryl S-200	2.73	0.14	0.383	10.1	27.5	72	1800	45
6. Mono Q	0.395	0.182	0.072	29.6	11.7	163	4080	19

Fig. 1. Chromatography of *E. coli* 3-dehydroquinase on Sephacryl S-200 (step 5 of purification scheme)

Enzyme from step 4 (40 units in 43 ml) was concentrated and applied to a column of Sephacryl S-200 (85 cm $\times$ 2.1 cm) and eluted as described in 'Materials and methods' (enzyme pool fractions 116–125). $\circ$, protein (mg/ml); $\bullet$, 3-dehydroquinase activity (units/ml); Δ , specific activity (units/mg of protein).

These three steps together resulted in 426-fold purification in 65% yield. After the gel filtration step the elution position of the peak of 3-dehydroquinase activity still did not correspond exactly to either of the main protein peaks (Fig. 1) although polyacrylamide-gel electrophoresis in the presence of SDS showed that the enzyme pool contained only two major species of polypeptide chain (Fig. 2, track D). The final purification step was chromatography on a Mono Q column (Fig. 3).

The purified enzyme showed a single band on polyacrylamide-gel electrophoresis in the presence of SDS (Fig. 2, track E). It also showed a single band on simple polyacrylamide gel electrophoresis (gel not shown). The specific activity of the purified enzyme was 163 units/mg of protein. After the DEAE-Sephacel step the partially purified enzyme was found to be stable for at least 2 months when stored in the same buffer that was used to store the final purified enzyme.

The subunit M_r of *E. coli* 3-dehydroquinase was estimated to be 29000 by polyacrylamide-gel electrophoresis in the presence of SDS. The native enzyme had an apparent M_r of 52000 when subjected to gel filtration

Fig. 2. Purification of 3-dehydroquinase from *E. coli*

This 12.5% polyacrylamide gel run in the presence of SDS and stained by the silver method monitors the purification of 3-dehydroquinase. The numbers on the right-hand side refer to the positions of marker proteins used for M_r calibration (for details see 'Materials and methods'). Track A, crude extract (4 μ g of protein); track B, 55–75% $(\text{NH}_4)_2\text{SO}_4$ fraction (4 μ g); track C, enzyme eluted from DEAE-Sephacel column (1.6 μ g); track D, enzyme eluted from Sephacryl S-200 column (0.25 μ g); track E, enzyme eluted from the Mono Q column (0.56 μ g).

on Sephacryl S-200. The native M_r estimated by gel filtration on a TSK G3000SW column was 58000. These data suggest that *E. coli* 3-dehydroquinase is a simple dimeric enzyme.

Comparison with the 3-dehydroquinase activity of the *N. crassa arom* multifunctional enzyme

The 3-dehydroquinase activity of the *N. crassa arom* multifunctional enzyme is completely inactivated by incubation with the substrate 3-dehydroquinase and sodium borohydride as described in 'Materials and methods'. The *E. coli* enzyme is completely inactivated by incubation under the same conditions, while control experiments involving incubation in the absence of substrate lead to no detectable inactivation of either enzyme.

A plot of $\log V_{\max}$ versus pH for the 3-dehydroquinase activity of the *N. crassa arom* multifunctional enzyme is shown in Fig. 4. This plot clearly indicates that a group with a pK_a of 6.3 is required in a deprotonated form for maximal activity. A very similar $\log V_{\max}$ versus pH plot

Fig. 3. Chromatography of *E. coli* 3-dehydroquinase on Mono Q (step 6 of purification scheme)

Enzyme from step 5 was applied to a column of Mono Q and eluted with a three-step linear gradient of NaCl (20–300 mM) in 20 mM-Tris/HCl (pH 7.5) as described in 'Materials and methods'. —, A_{280} ; ●, 3-dehydroquinase activity (units/ml); ---, gradient profile.

Fig. 4. pH-activity profile for the 3-dehydroquinase activity of the *N. crassa arom* multifunctional enzyme

The upper line (●) is a plot of $\log V_{\max}$ versus pH and the lower line (□) indicates enzyme stability versus pH. The assays were carried out at 25 °C. For the enzyme stability experiments samples were incubated at the specified pH for 5 min at 25 °C and then assayed at pH 7.0. A series of constant ionic strength buffers ($I = 0.5$) was used for the $V_{\max}$ determinations: citrate/phosphate for pH range 5–7, Tris/HCl, pH 7.5–8 and sodium carbonate/bicarbonate, pH 8.5–9.5.

is obtained for the *E. coli* enzyme (results not shown); in this case the pK_a of the essential group is 6.1.

The histidine-directed reagent diethylpyrocarbonate inhibits both 3-dehydroquinases under the conditions described in 'Materials and methods'. The inactivation is pseudo-first-order and in the presence of 1 mM reagent the half-life of *E. coli* 3-dehydroquinase is 23 min and the half life of the *arom* 3-dehydroquinase is 3.9 min. Secondary plots of the first order inactivation rate constants against diethylpyrocarbonate concentration

Table 2. Some kinetic parameters for the two 3-dehydroquinases

All the assays were carried out in 50 mM-potassium phosphate buffer at pH 7.0 and 25 °C. The K_m and K_i values were estimated as described in 'Materials and methods'. In a separate experiment carried out in 100 mM-Tris/HCl buffer, pH 7.5, K_m values of 26 μ M and 73 μ M were obtained for the *N. crassa arom* multifunctional enzyme and the *E. coli* enzyme respectively.

Kinetic parameter	Value (mM) for:	
	<i>N. crassa arom</i> 3-dehydro- quinase	<i>E. coli</i> 3-dehydro- quinase
K_m in 100 mM-potassium phosphate buffer	0.005	0.018
K_i for chloride	13	17
K_i for acetate	35	102
K_i for succinate	21	74
K_i for D-(+)-tartrate	9	21

are linear and allow the calculation of the second order rate constant for the reaction of the enzymes with the inhibitor. The values obtained are 30 $M^{-1} \cdot \text{min}^{-1}$ for *E. coli* 3-dehydroquinase and 180 $M^{-1} \cdot \text{min}^{-1}$ for the *arom* 3-dehydroquinase. The substrate 3-dehydroquininate protects both enzymes against inactivation.

The two biosynthetic 3-dehydroquinases show very similar susceptibilities to inhibition by simple anions; some typical results are presented in Table 2. Chloride, acetate, succinate and tartrate are all competitive inhibitors of both enzymes with K_i values in the mM range. For this reason it is best to use phosphate-containing buffers for measurement of the enzyme activities. The K_m for *E. coli* 3-dehydroquinase in 100 mM-potassium phosphate buffer, pH 7.5, is 18 μ M while the K_m for the *arom* 3-dehydroquinase under the same conditions is 5 μ M.

DISCUSSION

Butler *et al.* (1974) first reported that very crude preparations of *E. coli* 3-dehydroquinase were inhibited by treatment with the substrate 3-dehydroquininate and NaBH_4 . This observation led to the proposal that for the *E. coli* enzyme the first step of the 3-dehydroquinase reaction involved the formation of an imine intermediate between the keto group of 3-dehydroquininate and the ϵ -amino group of a lysine at the active site of the enzyme. Our results with the purified *E. coli* enzyme confirm this earlier work and also establish that the *N. crassa* biosynthetic 3-dehydroquinase works in a similar manner. The involvement of lysine has recently been confirmed by the isolation of a single radiolabelled tryptic peptide following inactivation of the *N. crassa* enzyme with NaB^3H_4 and 3-dehydroquininate (S. Chaudhuri, J. E. Walker, & J. R. Coggins, unpublished work).

A mechanism for the action of 3-dehydroquinase has been proposed by Walsh (1979) which in addition to the lysine side chain involved in imine formation also requires a basic group for proton abstraction. The pH-activity studies reported here demonstrate that both the *E. coli* and *N. crassa* 3-dehydroquinases require groups

with pK_a values near to 6, in a deprotonated form, for maximal activity. The experiments with diethylpyrocarbonate provide evidence that for both enzymes this group is the imidazole side chain of a histidine residue.

In spite of their obvious differences in quaternary structure the two biosynthetic 3-dehydroquinases thus appear to work by very similar mechanisms and to have similar arrangements of amino acid side chains in their active sites. Further evidence that the enzymes are structurally similar is provided by the experiments with simple competitive inhibitors (Table 2).

The fact that *E. coli* 3-dehydroquinase is a simple oligomeric protein contrasts with the situation found in *N. crassa* (Lumsden & Coggins, 1977, 1978), *Aspergillus nidulans* (Kinghorn & Hawkins, 1982), *Saccharomyces cerevisiae* (Larimer *et al.*, 1983) and *Schistosaccharomyces pombe* (Nakanishi & Yamamoto, 1984) where the biosynthetic enzyme is known to be part of the pentafunctional *arom* polypeptide chain. We have suggested, on the basis of limited proteolysis and genetic evidence and from knowledge of the sizes of the polypeptide chains, that the *N. crassa arom* multifunctional enzyme consists of five functional domains each structurally related to one of the corresponding *E. coli* monofunctional enzymes (Chaudhuri & Coggins, 1985; Coggins *et al.*, 1985). This hypothesis implies that each functional domain of the *N. crassa arom* multifunctional enzyme should work by a similar mechanism and have a similar arrangement of amino acid side chains in its active site as the corresponding monofunctional *E. coli* enzyme. The availability of a homogeneous preparation of *E. coli* 3-dehydroquinase has allowed us to test this hypothesis directly for one of the functional domains of the *N. crassa arom* multifunctional enzyme. A direct comparison of the catalytic properties of *E. coli* 3-dehydroquinase with those of the 3-dehydroquinase component of the *N. crassa arom* multifunctional enzyme demonstrates clearly that these two enzymes are mechanistically and structurally similar

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