The purification and characterization of 3-dehydroquinase from *Streptomyces coelicolor*

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The enzyme 3-dehydroquinase was purified over 4000-fold to homogeneity from *Streptomyces coelicolor*. The subunit M_r estimated from polyacrylamide-gel electrophoresis in the presence of SDS was 16000. The native M_r estimated by gel filtration on a Superose 6 column was 209000, indicating that the enzyme is a large oligomer. The enzyme was found to be extremely thermostable. This stability, along with the structural and kinetic properties of the enzyme, suggest that it is very similar to the quinate-inducible 3-dehydroquinase found in *Neurospora crassa* and *Aspergillus nidulans*. This similarity was confirmed by direct N-terminal sequencing.

INTRODUCTION

The shikimate pathway is the biosynthetic route by which plants and micro-organisms synthesize the aromatic amino acids [1,2]. The enzyme 3-dehydroquinase (EC 4.2.1.10) is the third enzyme in this seven-step pathway and catalyses the conversion of 3-dehydroquininate into 3-dehydroshikimate. In *Escherichia coli* 3-dehydroquinase is a dimeric protein of subunit M_r 27000 [3], whereas in fungi and plants the activity is part of larger multifunctional proteins [4–7]. Where sequencing, chemical modification and kinetic data are available, it is clear that the biosynthetic enzymes show marked similarities [8–10] and are closely related.

In contrast with the biosynthetic 3-dehydroquinases (class I 3-dehydroquinases), there exist a second class of proteins showing 3-dehydroquinase activity. These enzymes form part of the inducible catabolic quinate pathway used by some soil micro-organisms for the oxidation of quinate to protocatechuate, which can then be further metabolized via the β -oxoadipate pathway.

The biosynthetic and catabolic 3-dehydroquinases have been extensively characterized in two fungal species *Neurospora crassa* and *Aspergillus nidulans*. In these two micro-organisms sequence information clearly shows that the biosynthetic type I 3-dehydroquinases are not homologous to the type II catabolic enzymes [10–12]. The type I enzymes form an autonomous structural region (domain) of the *arom* pentafunctional polypeptide, and are similar in size (domain M_r 27000) and catalytic properties to the corresponding monofunctional *E. coli* enzyme [3,8,10,13]. In contrast, the type II enzymes are large multimeric proteins (probably dodecamers), consisting of identical monofunctional subunits of M_r 16000–18000 [12–14].

In the present paper we report that the biosynthetic 3-dehydroquinase of *Streptomyces coelicolor* is not a typical biosynthetic type I enzyme, but instead more closely resembles the catabolic type II enzymes previously found only in fungi.

MATERIALS AND METHODS

Reagents

All the reagents except those specified below were obtained from Boehringer Corp., Lewes, East Sussex, U.K., Sigma Chemical Co., Poole, Dorset, U.K., or BDH Chemicals, Poole, Dorset, U.K. DEAE-Sephacel was obtained from Pharmacia, Milton Keynes, Bucks., U.K. Ammonium 3-dehydroquininate was prepared by the method of Grewe & Haendler [15].

Growth of cells

S. coelicolor strain JI 3456 was grown for 96 h at 30 °C on a rotary shaker (180 rev./min). Cultures were grown in 2-litre flasks containing an inorganic medium (400 ml) supplemented with 0.4 % (w/v) glucose as the sole carbon and energy source [16].

Assay of 3-dehydroquinase activity

Formation of 3-dehydroshikimate was monitored by measuring the increase in A_{234} (ϵ 1.2×10^4 M⁻¹·cm⁻¹). Assays were carried out in a final volume of 1 ml at 30 °C in 50 mM-Tris/HCl buffer, pH 8.0. The dehydroquininate concentration was 0.5 mM for standard assays and in the range 0.1–2.5 mM for the determination of K_m . One unit of enzyme activity is defined as the amount catalysing the conversion of 1 μ mol of substrate/min.

Protein determination

Protein was determined by the method of Bradford [17], with bovine serum albumin as standard.

Purification of 3-dehydroquinase

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

Step 1: extraction and centrifugation. A 185 g batch (wet wt.) of *S. coelicolor* cells was suspended in 35 ml of 100 mM-potassium phosphate buffer, pH 7.0, containing 5 mM-EDTA, 1.2 mM-phenylmethanesulphonyl fluoride

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and 0.4 mM-dithiothreitol (buffer A) and broken by two passages through a French pressure cell (98 MPa internal pressure). This material was then centrifuged at 100 000 *g* for 1 h. 3-Dehydroquinase was purified from the resulting supernatant.

Step 2: fractionation with $(\text{NH}_4)_2\text{SO}_4$. The crude extract was diluted to 250 ml and adjusted carefully to 30% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ (175 g/l). The mixture was stirred for 20 min and the precipitate was then removed by centrifugation at 28 000 *g* for 30 min. The supernatant was adjusted to 45% saturation with $(\text{NH}_4)_2\text{SO}_4$ by adding a further 93 g/l and stirred for 20 min. The precipitated protein was collected by centrifugation at 28 000 *g* for 30 min, resuspended in buffer A and dialysed overnight against 2 litres of 50 mM-Tris/HCl buffer, pH 7.5, containing 1.2 mM-phenylmethanesulphonyl fluoride, 0.4 mM-dithiothreitol and 30 mM-KCl (buffer B).

Step 3: DEAE-Sephacel chromatography. The dialysed material was loaded on to a column of DEAE-Sephacel (bed volume 60 ml) equilibrated in buffer B. The column was washed with buffer until the A_{280} of the eluate had fallen to less than 0.1. The column was then eluted with a 500 ml linear gradient of 30–500 mM-KCl in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.4 mM-dithiothreitol (flow rate 50 ml/h). Fractions (5 ml) containing high 3-dehydroquinase activity were pooled and dialysed overnight against 1 litre of 50 mM-Tris/HCl buffer, pH 7.5, containing 0.4 mM-dithiothreitol and 1 M- $(\text{NH}_4)_2\text{SO}_4$ (buffer C).

Step 4: hydrophobic-interaction chromatography on phenyl-Superose. Steps 4 and 5 were carried out at room temperature with a Pharmacia f.p.l.c. system. The enzyme from step 3 was applied to a phenyl-Superose hydrophobic-interaction column in buffer C and eluted with a linear decreasing gradient of $(\text{NH}_4)_2\text{SO}_4$ (1.0–0 M). The flow rate was 0.5 ml/min, and 0.5 ml fractions were collected. The fractions containing the highest 3-dehydroquinase activity were pooled and dialysed against 1 litre of 25 mM-Tris/HCl buffer, pH 7.5, containing 0.4 mM-dithiothreitol (buffer D).

Step 5: chromatography on Mono Q. The enzyme was applied to a Mono Q anion-exchange column and eluted with a linear gradient of 0–500 mM-KCl in buffer D (flow rate 1 ml/min; 0.5 ml fractions). Fractions containing 3-dehydroquinase activity were pooled and dialysed against 50 mM-Tris/HCl buffer, pH 7.5, containing 0.4 mM-dithiothreitol and 50% (v/v) glycerol, for long-term storage at -20°C .

Step 6: heat treatment. Protein required for micro-sequencing was subjected to a final purification step. The active fractions from the Mono Q chromatography step were pooled and heated at 85°C for 10 min. After cooling and centrifugation at 14 000 *g* for 10 min, the supernatant was removed and dialysed exhaustively against 0.5% (w/v) NH_4HCO_3 and freeze-dried.

PAGE

Electrophoresis in the presence of SDS was performed by the method of Laemmli [18], with a 3% poly-

acrylamide stacking gel and a 15% polyacrylamide running gel. Gels were stained for protein with AgNO_3 [19].

Automatic amino acid sequence determination

Samples of 3-dehydroquinase estimated at 0.9 and 2.8 nmol were sequenced on an Applied Biosystems 470A protein sequencer with on-line detection of amino acid phenylthiohydantoins by a 120A analyser.

The first analysis gave a 27-amino acid-residue sequence. This was confirmed by a second run, which extended the sequence to 34 amino acid residues. The repetitive yields were 93% for both analyses. Initial yields were very low (5%), suggesting losses of sample on reconstitution for sequencing.

Determination of native M_r

The native molecular M_r of the *S. coelicolor* 3-dehydroquinase was determined by gel filtration on a Superose 6 column in a Pharmacia f.p.l.c. apparatus. The column was eluted with 50 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-KCl and 0.4 mM-dithiothreitol (flow rate 0.5 ml/min, fraction size 0.25 ml). The eluate was monitored at 280 nm and the column was calibrated with the following proteins: horse heart ferritin (M_r 440 000), rabbit muscle pyruvate kinase (M_r 240 000), pig heart lactate dehydrogenase (M_r 140 000), rabbit muscle hexokinase (M_r 100 000), chicken ovalbumin (M_r 45 000) and horse heart cytochrome *c* (M_r 12 500).

RESULTS AND DISCUSSION

Enzyme purification and subunit structure

The purification of the *S. coelicolor* 3-dehydroquinase is summarized in Table 1. Three chromatographic steps were required to obtain enzyme that was over 75% pure and stable on storage at -20°C : ion-exchange chromatography on DEAE-Sephacel, hydrophobic-interaction chromatography on phenyl-Superose and finally a second anion-exchange step on Mono Q. Only one peak of 3-dehydroquinase activity was detected at each of these steps. The enzyme was purified nearly 4000-fold in 17% yield from the crude extract by this procedure.

It was noted that the enzyme was extremely thermostable, and heating *S. coelicolor* crude extracts at 70°C for 10 min did not result in any significant loss of activity. This property was used to obtain electrophoretically homogeneous enzyme suitable for micro-sequencing. The heat step removed nearly all the remaining contaminating polypeptides. The purified enzyme showed a single band on PAGE in the presence of SDS (Fig. 1) with a mobility corresponding to a subunit M_r of 16 000. The M_r of the native enzyme was 209 000 as judged by gel-permeation chromatography on a Superose 6 column (results not shown). This result suggests that the native enzyme is an oligomer most likely containing 12 subunits.

The heat-stability of the enzyme in crude extracts and the detection of only one peak of activity at each of the column-chromatography steps provides strong evidence that the enzyme purified in this study constitutes all of the 3-dehydroquinase activity in *S. coelicolor*. This is in accordance with the conclusions of Berlyn & Giles [20]. Their sucrose-density-centrifugation analysis of crude extracts indicated that *S. coelicolor* contained only one 3-dehydroquinase, with a native M_r of 134 000.

Table 1. Purification scheme for *S. coelicolor* 3-dehydroquinase

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	196	902	42	0.046	1	100
30–45 %-satn. $(\text{NH}_4)_2\text{SO}_4$	67	167	33.5	0.2	4.3	80
DEAE-Sephacel	50	20	24.5	1.22	26.6	58
Phenyl-Superose	1.5	1.2	17.8	14.9	323.5	42.5
Mono Q	1.0	0.04	7.3	183.2	3984	17.4

Fig. 1. SDS/PAGE of purified 3-dehydroquinase after heat treatment

This 15 % denaturing gel was stained with AgNO_3 . Track A, highly purified 3-dehydroquinase (3.5 μg); track B, M_r markers.

Kinetic properties

The apparent K_m for 3-dehydroquinase was determined from Lineweaver–Burk, Eadie–Hofstee and Hanes plots with highly purified enzyme that had not been heat-treated. The enzyme was assayed at 30 °C in 50 mM-Tris/HCl buffer, pH 8.0. All three plots gave a K_m value of approx. 650 μM . Enzyme activity was low in phosphate buffers and showed maximal activity in Tris/HCl buffer at pH 8.0. The *S. coelicolor* enzyme has a K_m value nearly 20 times that obtained for the *E. coli* 3-dehydroquinase when assayed under the same conditions. The Cl^- anion inhibits the monofunctional 3-dehydroquinase of *E. coli* and the 3-dehydroquinase activity of the *arom* penta-functional enzyme from *N. crassa* [3], as well as the 3-dehydroquinase activity associated with pea shoot chloroplast extracts [9]. Cl^- was a competitive inhibitor in

each case, with K_i values of 17 mM, 13 mM and 50 mM respectively. Cl^- had no significant effect on the activity of the *S. coelicolor* 3-dehydroquinase at concentrations up to 250 mM-KCl when assayed at 0.5 mM-3-dehydroquinase.

Enzyme from step 5 (1.8 munits) in 100 μl of 100 mM-Tris/HCl buffer, pH 8.0, containing 0.4 mM-dithiothreitol was completely inactivated by adding ammonium dehydroquinase to a final concentration of 2 mM, followed by 50 μl of 5 M- NaBH_4 in 50 mM- NaOH . Control experiments involving incubation with NaBH_4 in the absence of substrate resulted in no detectable loss of activity. This result is consistent with the proposal that the first step of the 3-dehydroquinase reaction involves the formation of an imine intermediate between the oxo group of 3-dehydroquinase and the enzyme [3,21].

Primary-structure analysis

The *N*-terminal amino acid could not be identified, but the next 33 residues were assigned, with the exception of residue 32. The *N*-terminal amino acid sequence of the *S. coelicolor* 3-dehydroquinase was compared with the amino acid sequences of the other characterized 3-dehydroquinases. The sequence shows strong similarity to the *N*-terminal region of the inducible catabolic 3-dehydroquinase from *N. crassa* and *A. nidulans* (Fig. 2): 17 of the 34 amino acid residues sequenced matched identically with residues in both the *N. crassa* and the *A. nidulans* inducible enzymes.

Comparison of the *S. coelicolor* 3-dehydroquinase with other characterized enzymes

With respect to heat-stability, subunit size, oligomeric structure and *N*-terminal sequence the *S. coelicolor* 3-dehydroquinase is very similar to the type II quinate-pathway 3-dehydroquinase found in *N. crassa* and *A. nidulans*, but is quite different from the type I enzymes.

The possibility that *S. coelicolor* possesses a quinate pathway, constitutive or inducible, was investigated. We were unable to detect the presence of quinate dehydrogenase activity in cells grown on the defined minimal medium, and we were unable to detect growth on a minimal medium supplemented with quinate as the sole carbon source. Growth on *p*-hydroxybenzoate (a compound that can induce the quinate pathway in *A. calcoaceticus* [22]) was observed, but quinate dehydrogenase was not detectable and the specific activity of 3-dehydroquinase was no higher than the value obtained for cells grown on glucose. These data indicate that *S. coelicolor* has a functional β -oxoadipate pathway but lacks the ability to metabolize quinate.

S. coelicolor is the first micro-organism to be studied

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      *RSLANAPIMILNGPNLNLLGQROPEIYGSD*LA  S. coelicolor
      MASPRHILLINGPNLNLLGTREPOIYGSTTLH  N. crassa
      MEKSIILLINGPNLNLLGTREPHIYGSTTLLS  A. nidulans

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Fig. 2. Alignment of the *N*-terminal sequence of the *S. coelicolor* 3-dehydroquinase with the sequence of the quinate-inducible 3-dehydroquinases from *N. crassa* and *A. nidulans*

The sequences were aligned by eye. Identical amino acid residues are boxed, and asterisks indicate residues that could not be identified unambiguously from amino acid sequencing. The single-letter amino acid abbreviations are used.

that does not have a type I 3-dehydroquinase as part of its shikimate pathway. This may be a common feature of streptomycetes, and the findings presented in this paper could represent an important distinction between the structural organization of the shikimate pathway in streptomycetes and other organisms.

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