Evidence for a novel class of microbial 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase in *Streptomyces coelicolor* A3(2), *Streptomyces rimosus* and *Neurospora crassa*

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The tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthases from *Streptomyces coelicolor* A3(2), *Streptomyces rimosus* and *Neurospora crassa* have been purified to homogeneity. All three enzymes have a subunit *M_r* of 54 000. The *S. coelicolor* DAHP synthase was physically and kinetically characterized and the N-terminal amino acid sequence was obtained. The N-terminal amino acid sequence could not be obtained for the enzymes from *S. rimosus* and *N. crassa*, their N-termini apparently being blocked. However, following proteolytic digestion, internal amino acid sequences were obtained from both enzymes. A comparison with the known DAHP synthase sequences indicated that these DAHP synthases are unrelated to other microbial DAHP synthase sequences but are similar to plant DAHP synthases. Up until now, two distinct classes of DAHP synthase have been described, one comprising exclusively enzymes from plants, the other restricted to enzymes from micro-organisms. These studies indicate that the class containing the plant DAHP synthases also contains enzymes from a microbial eukaryote and from several bacteria.

Keywords: 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, shikimate pathway, *Streptomyces* species, *Neurospora crassa*

INTRODUCTION

In micro-organisms and plants, chorismate is the common metabolic precursor of the aromatic amino acids, vitamins E and K, folic acid, ubiquinone, plastoquinone, enterochelin and various secondary metabolites. This compound is synthesized by way of the seven-step shikimate pathway (Haslam, 1974, 1993; Weiss & Edwards, 1980). Other organisms lack this biosynthetic ability and require a supplementary dietary intake of aromatic amino acids.

The first step in the shikimate pathway involves the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) to form the phosphorylated 7-carbon keto sugar acid, 3-deoxy-D-arabino-heptulosonate-

7-phosphate (DAHP) and inorganic phosphate. This is achieved by the action of DAHP synthase (EC 4.1.2.15) (Srinivasan & Sprinson, 1959).

Branched, purely biosynthetic pathways exhibit a diversity of control mechanisms in different species. In the biosynthetic pathway for aromatic amino acids, different control and organization of the constituent enzymes are well-documented (Herrmann, 1983; Bentley, 1990). Carbon flow through the shikimate pathway is controlled at the first step by modulation of DAHP synthase activity either by feedback inhibition or by transcriptional regulation. Diverse patterns of control have been described for DAHP synthases from different organisms (Bentley, 1990). Different micro-organisms possess from one to three isoenzymes which are inhibited by feedback control, by end-products or intermediates of the pathway, or a combination of the two. In exceptional cases, the enzymes may be unregulated.

Nucleotide sequences for microbial DAHP synthases have been obtained for the three *Escherichia coli* isoenzymes

Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; E4P, erythrose 4-phosphate; PEP, phosphoenolpyruvate.

The PIR accession numbers for the *Streptomyces coelicolor*, *Streptomyces rimosus* and *Neurospora crassa* partial protein sequences reported in this paper are P80574, P80575 and P80576, respectively.

(Davies & Davidson, 1982; Schultz *et al.*, 1984; Ray *et al.*, 1988), the two *Saccharomyces cerevisiae* isoenzymes (Paravicini *et al.*, 1988; Kunzler *et al.*, 1992), *Candida albicans* (Pereira & Livi, 1993), *Salmonella typhimurium* (Muday & Herrmann, 1990), *Corynebacterium glutamicum* (Chen *et al.*, 1993) and for part of the gene from *Erwinia herbicola* (Xia *et al.*, 1993). They exhibit a striking level of sequence similarity, suggesting that they are derived from a common ancestral sequence.

cDNA clones encoding DAHP synthases have been isolated from potato (Dyer *et al.*, 1990; Zhao & Herrmann, 1992), tobacco (Wang *et al.*, 1991), *Arabidopsis thaliana* (Keith *et al.*, 1991) and tomato (Gorlach *et al.*, 1993). By varying assay conditions, two distinct plant isoforms possessing discrete divalent metal cation requirements can be identified (for review, see Mousdale & Coggins, 1993). All the plant sequences display a high degree of similarity (Keith *et al.*, 1991; Gorlach *et al.*, 1993) and are unrelated to the microbial sequences (Dyer *et al.*, 1990).

The genus *Streptomyces* comprises a group of Gram-positive obligately aerobic bacteria characterized by their morphological and metabolic capabilities (for review, see Chater & Hopwood, 1993). The antibiotics produced by streptomycetes are of considerable interest because of their medical, veterinary and agricultural importance (Demain, 1983).

The shikimate pathway enzymes, and DAHP synthase in particular, have not been extensively studied in *Streptomyces*, and no amino acid sequence data have been obtained for any *Streptomyces* DAHP synthase. An understanding of the regulation of the pathway would be of interest in determining factors influencing provision of precursors for antibiotic biosynthesis, given that many antibiotic structures are derived, in part, from the shikimate pathway. With this objective, a study on the DAHP synthases from two species of *Streptomyces* was undertaken.

In this paper, we report the purification and characterization of a single tryptophan-sensitive DAHP synthase activity from *Streptomyces coelicolor* A3(2) and the determination of its N-terminal amino acid sequence. In addition, internal amino acid sequences have been obtained for DAHP synthases of *Streptomyces rimosus* and *Neurospora crassa*. A comparison with the published DAHP synthase sequences indicates that the *Streptomyces* and *Neurospora* DAHP synthases are unrelated to other microbial DAHP synthase sequences but are similar to plant DAHP synthases.

METHODS

Strains and growth of cells. *S. coelicolor* A3(2) J13456 (SCP1^{NF}, SCP2⁻) was provided by Professor D. A. Hopwood, John Innes Institute, Norwich, UK. Cells were grown on a minimal medium comprising inorganic salts supplemented with 0.4% (w/v) glucose as described by Bramwell *et al.* (1993).

S. rimosus strain 4018 was provided by Pfizer (Sandwich, UK) and was grown as described by Stuart & Hunter (1993).

Freeze-dried *N. crassa* mycelia (strain 74-OR23-1A, FGSC 987; Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, CA, USA) were obtained as described by Lumsden & Coggins (1977).

Assay of DAHP synthase activity. DAHP synthase activity was measured using either a coupled continuous or a direct continuous spectrophotometric assay (Mousdale & Coggins, 1993). Assays were performed at 30 °C in a total volume of 1 ml. For the coupled assay, activity was measured by using the second to fourth enzymes of the shikimate pathway to couple the formation of DAHP to the oxidation of NADPH. The assay cocktail contained 50 mM bis-tris propane/HCl buffer (pH 7.0), 0.5 mM PEP, 1.0 mM E4P, 0.1 mM NADPH, 10 µM NAD⁺, 0.1 units of *E. coli* dehydroquinase, 0.05 units of *E. coli* dehydroquinase and 0.05 units of *E. coli* shikimate dehydrogenase. The oxidation of NADPH was monitored at 340 nm ($\epsilon_{340} = 6.18 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) using a Philips PU8720 spectrophotometer. The coupling enzymes dehydroquinase synthase, 3-dehydroquinase and shikimate dehydrogenase were purified from overproducing *E. coli* strains as described by Medhi *et al.* (1987), Chaudhuri *et al.* (1987b) and Chaudhuri *et al.* (1987a), respectively. For the direct assay (Schoner & Herrmann, 1976), the standard assay mixture contained 50 mM bis-tris propane/HCl buffer (pH 7.0), 0.5 mM PEP and 1.0 mM E4P. (One unit of enzyme activity is defined as the amount catalysing the conversion of 1 µmol substrate to product min⁻¹.)

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

Small-scale purification of *S. coelicolor* A3(2) DAHP synthase

Unless otherwise stated, all manipulations following cell breakage were performed at 4 °C.

Step 1: extraction and centrifugation. A 70 g batch (wet weight) of *S. coelicolor* cells was suspended in 20 ml 100 mM potassium phosphate buffer (pH 7.0), containing 1.2 mM PMSF, 3.5 mM benzamidine and 0.4 mM DTT (buffer A) and broken by two passages through a French pressure cell. This material was then centrifuged at 100 000 g for 1 h. DAHP synthase was purified from the resulting cell-free extract.

Step 2: salt fractionation with (NH₄)₂SO₄. The crude extract was diluted to give a protein concentration of 10 mg ml⁻¹ and solid (NH₄)₂SO₄ was added to give 35% saturation (209 g l⁻¹). The mixture was stirred for 20 min and the precipitated proteins were removed by centrifugation at 28 000 g for 20 min. The supernatant was adjusted to 65% saturation with solid (NH₄)₂SO₄ (199 g l⁻¹) and stirred for 20 min. The precipitated protein was collected by centrifugation at 28 000 g for 20 min, resuspended in 15 ml 50 mM potassium phosphate buffer (pH 7.0), 1.2 mM PMSF, 3.5 mM benzamidine and 0.4 mM DTT (buffer B) and dialysed overnight against 2 l buffer B.

Step 3: anion-exchange chromatography on Mono Q. Steps 3, 4 and 5 were carried out at room temperature using a Pharmacia FPLC system. The dialysed material was loaded onto a preparative Mono Q anion-exchange column (volume 8 ml) equilibrated in buffer B. The column was washed with buffer until the A_{280} of the eluate had returned to the baseline value. Protein was eluted from the column with an 80 ml gradient of 0–0.5 M Na₂SO₄ in buffer B, flow rate 4 ml min⁻¹. Fractions (2 ml) were collected and assayed for DAHP synthase, and those containing the highest activity were retained.

Step 4: hydrophobic-interaction chromatography on phenyl-Superose. Retained fractions were made to 1 M (NH₄)₂SO₄ by the addition of solid (NH₄)₂SO₄ and applied to a phenyl-Superose hydrophobic-interaction column (volume 1 ml) equil-

ibrated in 50 mM potassium phosphate buffer (pH 7.0), 1.2 mM PMSF, 3.5 mM benzamidine, 0.4 mM DTT and 0.9 M $(\text{NH}_4)_2\text{SO}_4$ (buffer C). After the A_{280} value of the eluate had returned to the pre-loading value, a 0.9–0 M $(\text{NH}_4)_2\text{SO}_4$ gradient was run in a volume of 15 ml. The column was washed at 0.5 ml min⁻¹ and 0.5 ml fractions were collected. After assaying for DAHP synthase activity, appropriate fractions were retained. These fractions were desalted into 215 mM sodium phosphate buffer, pH 6.7, containing 0.4 mM DTT (buffer D) using a Pharmacia FPLC desalting column (G-50).

Step 5: anion-exchange chromatography on Mono Q. The partially purified material was loaded onto an analytical Mono Q anion-exchange column (volume 1 ml) equilibrated in buffer D. Following washing with buffer D, protein was eluted with a 30 ml linear gradient of 215–320 mM sodium phosphate. The flow rate was 1 ml min⁻¹ and 0.5 ml fractions were collected. Fractions containing the peak of DAHP synthase activity were pooled and retained.

Large-scale purification of *S. coelicolor* A3(2) DAHP synthase.

Step 1 (extraction and centrifugation) was carried out as described for the small-scale purification of *S. coelicolor* DAHP synthase, except that 0.2 mM PEP was included in the extraction buffer and a 200 g batch of cells was used. Step 2 [salt fractionation with $(\text{NH}_4)_2\text{SO}_4$], step 3 (hydrophobic-interaction chromatography on phenyl-Sepharose) and step 4 (anion-exchange chromatography on Mono Q) were carried out as described for the purification of the *S. rimosus* DAHP synthase (below). All buffers were supplemented with 0.2 mM PEP, 1.2 mM PMSF and 3.5 mM benzamidine.

Purification of *S. rimosus* DAHP synthase

Unless otherwise stated, all manipulations following cell breakage were performed at 4 °C.

Step 1: extraction and centrifugation. An 85 g batch (wet weight) of *S. rimosus* cells was suspended in 60 ml 100 mM bis-tris propane buffer (pH 7.0), containing 3.5 mM benzamidine, 0.4 mM DTT and 0.05 mg DNase ml⁻¹ (buffer E) and broken by two passages through a French pressure cell. The extract was then stirred on ice for 60 min to allow for digestion of genomic DNA. This material was then centrifuged at 100 000 g for 1 h. DAHP synthase was purified from the resulting cell-free extract.

Step 2: salt fractionation with $(\text{NH}_4)_2\text{SO}_4$. This was carried out as above for the small-scale purification of *S. coelicolor* DAHP synthase. In this instance, crude extract was diluted in buffer E and the precipitated protein was resuspended in 25 ml 25 mM bis-tris propane buffer (pH 7.5), containing 0.4 mM DTT (buffer F) and dialysed overnight against 2 × 2 l buffer F.

Step 3: anion-exchange chromatography on DEAE-Sephacel. This dialysate was centrifuged at 100 000 g for 20 min to remove precipitated protein and other debris, prior to application onto a DEAE-Sephacel anion-exchange column (volume 70 ml) equilibrated in buffer F. The column was washed with buffer F until the A_{280} of the eluate was less than 0.3. The column was then washed with buffer F containing 150 mM NaCl until the A_{280} of the eluate was again less than 0.3. Protein was then eluted with a gradient of 150–350 mM NaCl in 1 l buffer F (15 column volumes) and fractions (16 ml) were collected and assayed as before.

Step 4: hydrophobic-interaction chromatography on phenyl-Sepharose. The pooled fractions from step 3 were made to 0.8 M $(\text{NH}_4)_2\text{SO}_4$ and loaded onto a phenyl-Sepharose hydrophobic-interaction column (bed volume 10 ml) equilibrated in 50 mM bis-tris propane/HCl (pH 7.5), 0.7 M $(\text{NH}_4)_2\text{SO}_4$ and 0.4 mM DTT (buffer G). The column was washed with buffer G until the A_{280} of the eluate was less than 0.1. Protein was eluted from

the column with a 150 ml gradient of 0.7–0 M $(\text{NH}_4)_2\text{SO}_4$ in 50 mM bis-tris propane buffer (pH 7.5), containing 0.4 mM DTT, collecting 3 ml fractions. These were assayed for DAHP synthase activity and those containing the highest activity were retained and dialysed against 1 l 25 mM bis-tris propane/HCl (pH 7.5), 50 mM NaCl and 0.4 mM DTT (buffer H).

Step 5: anion-exchange chromatography on Mono Q. Steps 5 and 6 were carried out at room temperature using a Pharmacia FPLC system. The dialysate was applied to an analytical Mono Q anion-exchange column (volume 1 ml) equilibrated in buffer H. Following washing with buffer H, protein was eluted with a gradient of 150–300 mM NaCl in 20 ml buffer H. Fractions (0.5 ml) were collected and assayed as before.

Step 6: hydrophobic-interaction chromatography on phenyl-Superose. Retained fractions were made to 1.1 M $(\text{NH}_4)_2\text{SO}_4$ by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ and applied to a phenyl-Superose hydrophobic-interaction column (volume 1 ml) equilibrated in 50 mM bis-tris propane/HCl (pH 7.5), 0.4 mM DTT and 1.1 M $(\text{NH}_4)_2\text{SO}_4$ (buffer I). After the A_{280} value of the eluate had returned to the pre-loading value a 1.1–0 M $(\text{NH}_4)_2\text{SO}_4$ gradient was run in a volume of 15 ml and 0.5 ml fractions were collected. After assaying for DAHP synthase activity, appropriate fractions were retained and stored.

Purification of *N. crassa* tryptophan-sensitive DAHP synthase.

The enzyme was purified essentially as described by Nimmo & Coggins (1981a), except that at the initial stage a DEAE-cellulose (DE52) chromatography step was employed to remove contaminating proteases (Coggins *et al.*, 1987).

PAGE. Electrophoresis in the presence of SDS was performed by the method of Laemmli (1970), with a 3% (w/v) stacking gel and a 10% (w/v) running gel. After electrophoresis, gels were stained for protein using either Coomassie Brilliant Blue dye or the silver nitrate method (Wray *et al.*, 1981).

Determination of native M_r . Gel-permeation chromatography on a Superose 12 column was used to estimate the native M_r of the purified *S. coelicolor* enzyme. The column buffer was 50 mM potassium phosphate, pH 7.0, 0.4 mM DTT (flow rate = 0.3 ml min⁻¹, fraction size 0.3 ml). Protein was monitored at 280 nm. The column was calibrated with the following standard proteins: pig heart malate dehydrogenase (M_r 70 000), pig muscle lactate dehydrogenase (M_r 140 000), rabbit muscle aldolase (M_r 160 000), rabbit muscle pyruvate kinase (M_r 232 000) and horse apoferritin (M_r 440 000).

Protein and peptide microsequencing. Protein and peptide samples for sequencing were resolved by SDS-PAGE in a separating gel appropriate for the M_r values of the samples, electroblotted onto PVDF membrane and subjected to microsequencing (Matsudaira, 1987) using an Applied Biosystems 477A protein sequencer.

Preparation of peptides from purified enzymes

(a) *S. coelicolor* DAHP synthase. Purified enzyme was subjected to SDS-PAGE in a 10% (w/v) gel. The bands were visualized by staining with Coomassie Blue R-250 for 5 min, followed by destaining for 20 min. The band corresponding to DAHP synthase was excised and neutralized in two changes of buffer containing 125 mM Tris/HCl (pH 6.8), 0.1% SDS, 1 mM DTT. The band was stored at -20 °C in the same buffer until ready to use. Gel slices were loaded onto a second 18% (w/v) SDS gel, and the protein was digested with endoproteinase Glu-C (V8) during electrophoresis as described by Cleveland *et al.* (1977).

(b) *S. rimosus* and *N. crassa* DAHP synthases. Purified enzyme was digested with endoproteinase Lys-C. Digests were carried out under denaturing conditions at 37 °C with a ratio of substrate to

protease of 50:1 (w/w). The number and size of proteolytic fragments were analysed by SDS-PAGE throughout a time-course of digestion and appropriate conditions to yield peptides capable of resolution by SDS-PAGE for subsequent electroblotting to PVDF membranes and microsequencing were determined.

Amino acid sequence comparison analysis. Amino acid sequences were analysed by using the sequence analysis programs of the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

RESULTS AND DISCUSSION

Purification and characterization of the *S. coelicolor* DAHP synthase

This paper reports the purification to homogeneity of a single tryptophan-sensitive DAHP synthase from *S. coelicolor*. The small-scale purification is summarized in Table 1. The purified preparation was shown to be essentially electrophoretically homogeneous under both denaturing (Fig. 1) and non-denaturing conditions (data not shown). The purification, as judged by the final specific activity of 8.3 U mg^{-1} , was 2380-fold with an 8% yield (Table 1). This final specific activity was of a similar order to other homogeneous, purified, microbial and plant DAHP synthases (Sugimoto & Shiio, 1980; Nimmo & Coggins, 1981a; Pinto *et al.*, 1986; Paravicini *et al.*, 1989; Ray & Bauerle, 1991; Stuart & Hunter, 1993). No evidence was found during the purification to indicate the presence of more than one isoenzyme of DAHP synthase in *S. coelicolor*.

The subunit M_r of the purified enzyme, determined electrophoretically under denaturing conditions, was 54000 ± 2000 (Fig. 1) and the native M_r , as estimated by gel-permeation chromatography, was 107000 ± 10000 . This indicates that the native enzyme is a dimer.

The kinetic studies were carried out on enzyme purified to the penultimate stage using the small-scale procedure. At this stage, the enzyme was relatively stable and free of contaminating blank rates. Duplicate initial velocity values were determined as a function of the concentration of one substrate at a fixed saturating level of the other substrate (for PEP = 0.5 mM , for E4P = 1 mM) using the direct continuous assay. Mean apparent K_m values for

Fig. 1. Analysis of the purified *S. coelicolor*, *S. rimosus* and *N. crassa* DAHP synthases by SDS-PAGE. Tracks: 1, material from the final stage of the large-scale *S. coelicolor* DAHP synthase purification ($1.5 \mu\text{g}$); 2, purified *S. rimosus* DAHP synthase ($0.5 \mu\text{g}$); 3, purified *N. crassa* DAHP synthase ($0.5 \mu\text{g}$); 4, *S. coelicolor* DAHP synthase (small-scale purification). Tracks 1–3 were run together and stained with Coomassie Brilliant Blue and track 4 was run on a separate gel and stained with silver nitrate. The positions of migration of M_r markers are shown.

E4P of $195 \pm 5 \mu\text{M}$ and for PEP of $92 \pm 8 \mu\text{M}$ were derived from direct linear plots using regression analysis (MultiFit software package). PEP acted as a normal Michaelis–Menten substrate, showing hyperbolic saturation, whereas E4P acted as an inhibitor at concentrations above 1.1 mM , this inhibition being competitive with respect to PEP (data not shown).

A range of end-products and intermediates of the shikimate pathway were evaluated for their ability to

Table 1. Small-scale purification of *S. coelicolor* DAHP synthase

The results presented are for a typical purification starting from 70 g *S. coelicolor* cells.

Step	Total protein (mg)	Total activity (mU)	Specific activity (mU mg ⁻¹)	Yield (%)	Purification (-fold)
1. Crude extract	720	2520	3.5	100	1
2. (NH ₄) ₂ SO ₄ fractionation	288	1897	6.6	75	1.9
3. Mono Q chromatography	10	681	67.3	27	19
4. Phenyl-Superose chromatography	0.22	372	1690	15	483
5. Mono Q chromatography	0.024	200	8330	8	2380

Fig. 2. Saturation curve of tryptophan inhibition of the *S. coelicolor* DAHP synthase. DAHP synthase was assayed by the direct method with the addition of between 0 and 250 μ M tryptophan to assays containing final concentrations of 50 μ M PEP and 100 μ M E4P.

inhibit DAHP synthase. Only tryptophan was found to show any significant effect. At concentrations up to 1 mM, tyrosine and phenylalanine had no effect on enzyme activity, and similarly at concentrations up to 0.25 mM, anthranilate and *p*-aminobenzoate gave no inhibition. The partial feedback inhibition by tryptophan was saturable with an apparent K_i of 70 μ M (Fig. 2).

The influence of pH upon enzyme activity was investigated. Partially purified enzyme was assayed directly in duplicate under saturating conditions at different pH intervals in bis-tris propane/HCl buffer. Activity increased rapidly with increasing pH to an optimum between pH 7.0 and 7.4; further increases in pH led to a strong decrease in activity.

Upon manipulation and storage, the activity was found to be extremely labile. Overnight dialysis, against buffer B containing 10 mM EDTA at 4 °C, of aliquots redissolved from $(\text{NH}_4)_2\text{SO}_4$ pellets resulted in an 85% inactivation of the enzyme. Activity was also lost following overnight dialysis against Tris buffers at various pH values, possibly as a result of the metal-chelating properties of this buffer. Consequently, phosphate and bis-tris propane buffers were employed throughout the purification procedures and EDTA was omitted. It appears likely that this DAHP synthase requires a metal cofactor for activity, as described for other species (Stephens & Bauerle, 1991). In the absence of a reductant, activity was lost, and 0.4 mM DTT was included in all buffers. For the large-scale purification, one of the substrates (PEP) was included in all buffers. The polypeptide band responsible for the DAHP synthase activity was degraded upon storage. A co-purified lysine-specific protease was identified as being responsible following the sequencing of a band of M_r 54 000, the largest protein species corresponding to the DAHP synthase in the degraded material, which lacked the first five amino acids of the intact protein

(Table 3). The inclusion of PEP reduced the rate of proteolysis.

Microsequencing and sequence comparisons of protein and peptides from the *S. coelicolor* DAHP synthase

The specific activity of the material obtained by the large-scale purification (Fig. 1, track 1) was 2.6 U mg^{-1} , representing a 780-fold purification with a 15% yield. This final protein sample comprised several polypeptide species (Fig. 1, track 1). DAHP synthase activity correlated with the band of M_r 54 000, matching the size of the species identified as DAHP synthase by the small-scale procedure. An aliquot of the pool was subjected to SDS-PAGE and electroblotted to PVDF membrane. The N-terminal amino acid residues from positions 1 to 14 of the DAHP synthase were unambiguously assigned (Table 3). This material was also used to generate peptides from *in situ* proteolysis with V8 proteinase. Only one of the peptides (M_r 25 000) generated yielded useful sequence. Twenty-one residues from this peptide were sequenced and found to match the N-terminal sequence (Table 3).

This sequence did not show a significant match to DAHP synthase sequences in the databases, nor to any sequence lodged on the database. This was not unexpected as the first 41 residues of the otherwise highly homologous *E. coli* isoenzymes share only six amino acid identities (Ray *et al.*, 1988); similarly, the protein sequences deduced from plant cDNA clones are largely identical, except, again, in their N-terminal portions, which appear characteristic of chloroplast transit sequences (Dyer *et al.*, 1990; Keith *et al.*, 1991).

There are many other examples of proteins, otherwise highly conserved, displaying heterogeneity in amino acid sequence at their N-termini. Furthermore, it is difficult to assess the significance of amino acid alignments over short regions (e.g. 10–20 residues) and the probability of a match occurring by chance can be significant (Doolittle, 1981). In conclusion, these N-terminal amino acid sequence data alone cannot be used for the analysis of the relatedness of DAHP synthase species, and further internal peptide sequence information is required.

Purification and characterization of the *S. rimosus* DAHP synthase

A purification to apparent homogeneity of the tryptophan-sensitive DAHP synthase from *S. rimosus* was reported during the course of this work (Stuart & Hunter, 1993). With the instability of the *S. coelicolor* DAHP synthase activity rendering attempts to obtain internal amino acid sequence from purified enzyme technically difficult, the probability of obtaining reasonable amounts of enzyme from which to obtain internal amino acid sequence for DAHP synthase seemed greater using *S. rimosus* as a source of *Streptomyces* enzyme.

The enzyme was prepared in a novel six-step procedure yielding several hundred micrograms of material (Table

Table 2. Purification of *S. rimosus* DAHP synthase

The results presented are for a typical purification starting from 85 g *S. rimosus* cells.

Step	Total protein (mg)	Total activity (mU)	Specific activity (mU mg ⁻¹)	Yield (%)	Purification (-fold)
1. Crude extract	3300	9705	2.9	100	1
2. (NH ₄) ₂ SO ₄ fractionation	1512	13456	8.9	139	3.1
3. DEAE-Sephacel chromatography	74	6664	90	69	31
4. Phenyl-Sepharose chromatography	8.8	5932	674	61	229
5. Mono Q chromatography	0.96	4000	5422	41	1844
6. Phenyl-Superose chromatography	0.175	1928	11020	20	3750

2). The purified preparation was shown to be essentially homogeneous by electrophoresis under denaturing conditions (Fig. 1). The purification, as judged by the final specific activity of 11.02 U mg⁻¹, was 3750-fold with a 20% yield (Table 2). This final specific activity was 40% higher than that of 7.83 U mg⁻¹ reported previously (Stuart & Hunter, 1993). The subunit M_r was estimated to be 54000, similar to that obtained for the *S. coelicolor* DAHP synthase (54000: this study), the tryptophan-sensitive *N. crassa* DAHP synthase (52000: Nimmo & Coggins, 1981a; 54000: this study) and the mature plastidic potato DAHP synthase (53000: Dyer *et al.*, 1990). The previous subunit M_r value of 59000 (Stuart & Hunter, 1993) appears to be an overestimate.

This activity was found to be remarkably stable in comparison with the *S. coelicolor* enzyme. The inclusion of anti-protease agents was unnecessary and the addition of PEP in buffers did not improve the yield of enzyme activity.

Microsequencing and sequence comparisons of peptides from the *S. rimosus* DAHP synthase

An aliquot of the final pool was subjected to N-terminal amino acid sequence analysis following electrophoresis and electroblotting onto PVDF membrane. No sequence was obtained and it was concluded that the N-terminus was blocked. This material was also used to generate peptides following proteolysis with endoproteinase Lys-C. Many of the peptides did not yield sequence and were presumed to include the blocked N-terminus. Only one of the isolated peptides (M_r 10000) yielded useful sequence (Table 3). This sequence showed similarity to the plant DAHP synthases, with a sequence identity of 58% in a 19 amino acid overlap (Fig. 3). It appears likely that this peptide corresponds to the C-terminal region of the *S. rimosus* DAHP synthase. Translation of the plant nucleotide sequences from this region of alignment to the stop codons also results in peptide sequences of around M_r 10000. The peptide sequence was unrelated to any other previously published microbial DAHP synthase sequence.

Another sequence similar to the *S. rimosus* M_r 10000 peptide sequence was found in the database. The over-

lapping 19 amino acid sequence was found to match completely part of an amino acid sequence translated from a *Streptomyces lividans* nucleotide sequence (Fig. 3). The authors had submitted this DNA sequence to the database as part of a region of DNA which contained the sequence of a secretory signal peptide (L. L. Liaw and others; accession number L13767). The region upstream of the sequence encoding this secretory signal peptide appears to encode the C-terminal 146 amino acids of *S. lividans* DAHP synthase. The *S. lividans* sequence displayed the highest sequence identity with the potato (StshkB) and the *A. thaliana* 1 DAHP synthases (53% and 52%, respectively, of amino acid residues identical). It remains to be established whether the entire *S. lividans* gene corresponding to this partial nucleotide sequence encodes a functional DAHP synthase.

Microsequencing and sequence comparisons of peptides from the *N. crassa* DAHP synthase

N. crassa possesses three isoenzymes of DAHP synthase, each of which is subject to feedback inhibition by one of the three aromatic amino acids (Jensen & Nasser, 1968; Hoffmann *et al.*, 1972). Of these, only the tryptophan-sensitive isoenzyme has been purified to homogeneity and extensively characterized (Nimmo & Coggins, 1981a, b). We believed that it would be of interest and relevance to our studies of the DAHP synthases of *Streptomyces* to obtain amino acid sequence information from the *N. crassa* enzyme, as it shares with the *Streptomyces* enzymes the features of large subunit size and sensitivity to tryptophan.

The purification yielded enzyme with a final specific activity of 7.7 U mg⁻¹, representing a 2000-fold enrichment on the starting material. The purified preparation was shown to be essentially homogeneous by electrophoresis under denaturing conditions (Fig. 1) and the subunit M_r was estimated to be 54000 ± 2000.

An aliquot of the final pool was subjected to N-terminal amino acid sequence analysis following electrophoresis and electroblotting onto PVDF membrane. No sequence was obtained and it was concluded that the N-terminus was blocked. This material was then used to generate peptides using endoproteinase Lys-C. Several peptides

1									
50									
StshkB	MA	LSNTLSLSS	KSLVQSHLLH						
LeDHS1	MA	LSNTLSLSS	KSLVQSHLLH						
AtDHS1	MALSN	SSLSSTRSIY	GGDLSHRPSN	RQSSFTFHPA					
StshkA	MALSTSTT	NSLLPNKSLV	QNPPLPSPL	KNAFFSNNST	KTVRFVQPI				
LeDHS2	MALSTNTT	NSLLSNKSL	QNPPLSSPS	KNAFFSNKST	KTVRFVQPI				
NtDAHPS	MALSSSTTN	SLLPNKSQLV	QNPPLPSPL	KNVSFTTNST	KPVRFVQPI				
AtDHS2				MV	TLNASSPLTT				
100									
StshkB	NPLPQPRFSL	PPTTQHGRRH	PISAVHAAEP	SKTAVKQGW	SLDSWKTKKA				
LeDHS1	NPTPQPRFSL	PPTTQHGRRH	PISAVHAAEP	SKTAVKQGW	SLDSWKTKKA				
AtDHS1	VNTKPKSVNL	VTAVHAAEPA	RNAVSKEV	ASSSSGALKW	TPESWKLKKA				
StshkA	AVHSSDSNKN	PIVSDKPSKS	SPPAATATTA	PAPAVTKTEW	AVDSWKSCKA				
LeDHS2	AVHSSDSNKN	PIVSDKPSKS	SPPAATATTA	PAPAVTKTEW	AVDSWKSCKA				
NtDAHPS	AIHSSDSNKN	PIVSDKPSKS	SPPAATVTA	AATTVTKTEW	TVESWKSCKA				
AtDHS2	KSLPYRHAP	RRPISFSPVF	AVHSTDPKKS	TQSASASVKW	SLESWKSCKA				
150									
NtDAHPS	TLPPIVLPNE	IIRLREHLRD	VAQGKAPLLQ						
AtDHS1	QQLPEYDPEK	ELESVLKTL	MN...L.FAG.	ARS.E.K.GE	A.L.....				
AtDHS2	QQLPEYDPEK	ELESVLKTL	MN...L.FAG.	ARS.E.K.GE	A.L.....				
StshkA	QQLPDYPNAN	ELESVLKTL	AF...FAG.	ARN.E.R.A.	A.V.....				
LeDHS1	QQLPEYDPEK	ELESVLKTL	EF...FAG.	ARS.E.R.GE	A.M.R.....				
LeDHS2	QQLPEYDPEK	ELESVLKTL	EF...FAG.	ARS.E.R.GE	A.M.R.....				
NtDAHPS	QQLPEYDPEK	ELESVLKTL	EF...FAG.	ARS.E.R.GE	A.M.R.....				
AtDHS2	QQLPDYPNAN	DVDSVLQTL	SF...FAG.	ARK.EDK.GQ	A.M.Q.M.				
200									
StshkB	GGDCAESFKE	FNANNIRDTF	RILLQMSVVL	MFGGQVPVVK	VGRMAGQFAK				
LeDHS1	GGDCAESFKE	FNANNIRDTF	RILLQMSVVL	MFGGQVPVVK	VGRMAGQFAK				
AtDHS1	GGDCAESFKE	FNANNIRDTF	RILLQMSVVL	MFGGQVPVVK	VGRMAGQFAK				
StshkA	GGDCAESFKE	FNANNIRDTF	RILLQMSVVL	MFGGQVPVVK	VGRMAGQFAK				
LeDHS2	GGDCAESFKE	FNANNIRDTF	RILLQMSVVL	MFGGQVPVVK	VGRMAGQFAK				
NtDAHPS	GGDCAESFKE	FNANNIRDTF	RILLQMSVVL	MFGGQVPVVK	VGRMAGQFAK				
AtDHS2	GGDCAESFKE	FNANNIRDTF	RILLQMSVVL	MFGGQVPVVK	VGRMAGQFAK				
250									
StshkB	PRSDPLEEIN	GKVLPSYKGD	NINGDTFDEK	SRIPDPHRLI	RAYMQSAATL				
LeDHS1	PRSDPLEEIN	GKVLPSYKGD	NINGDTFDEK	SRIPDPHRLI	RAYMQSAATL				
AtDHS1	PRSDPLEEIN	GKVLPSYKGD	NINGDTFDEK	SRIPDPHRLI	RAYMQSAATL				
StshkA	PRSDPLEEIN	GKVLPSYKGD	NINGDTFDEK	SRIPDPHRLI	RAYMQSAATL				
LeDHS2	PRSDPLEEIN	GKVLPSYKGD	NINGDTFDEK	SRIPDPHRLI	RAYMQSAATL				
NtDAHPS	PRSDPLEEIN	GKVLPSYKGD	NINGDTFDEK	SRIPDPHRLI	RAYMQSAATL				
AtDHS2	PRSDPLEEIN	GKVLPSYKGD	NINGDTFDEK	SRIPDPHRLI	RAYMQSAATL				
300									
NtDAHPS	YOETVDR	IQEMLRPMHX							
AtDHS1	NLLRAFATGG	YAAQQRVTEW	NLDFVENCEQ	GDR...L.AH.	YD.A.G..AA				
AtDHS2	NLLRAFATGG	YAAQQRVTEW	NLDFVENCEQ	GDR...L.AH.	YD.A.G..AA				
StshkA	NLLRAFATGG	YAAQQRVTEW	NLDFVENCEQ	GDR...L.AH.	YD.A.G..AA				
LeDHS1	NLLRAFATGG	YAAQQRVTEW	NLDFVENCEQ	GDR...L.AH.	YD.A.G..AA				
LeDHS2	NLLRAFATGG	YAAQQRVTEW	NLDFVENCEQ	GDR...L.AH.	YD.A.G..AA				
NtDAHPS	NLLRAFATGG	YAAQQRVTEW	NLDFVENCEQ	GDR...L.AH.	YD.A.G..AA				
AtDHS2	NLLRAFATGG	YAAQQRVTEW	NLDFVENCEQ	GDR...L.AH.	YD.A.G..AA				
350									
NtDAHPS	IGADQNEK	L	STVELFTSHE	GLLLEYEPL	TTXLNAPSQ				
StshkB	A.LTVDHPI	M	TDFW...	C...P..QA.	REDSTSGLF	YDCSAHMMVW			
LeDHS1	A.LTVDHPI	M	TDFW...	C...P..QA.	REDSTSGLF	YDCSAHMMVW			
AtDHS1	C.LGTDHPI	M	TDFW...	C...P..QA.	REDSTSGLF	YDCSAHMMVW			
StshkA	A.LTMDHPI	M	K.T.FW...	C...P..QS.	RRDSTSGLY	YDCSAHFLWV			
LeDHS2	A.LTMDHPI	M	K.T.FW...	C...P..QS.	RRDSTSGLY	YDCSAHFLWV			
NtDAHPS	A.LTVDHPI	M	K.T.FW...	C...P..QS.	RRDSTSGLY	YDCSAHFLWV			
AtDHS2	A.LTSAHPI	M	T.T.FW...	C...P..QA.	REDSTSGLY	YDCSAHMLWV			
400									
S.lividans	GERTRQLDGA	HVEFLRGVAN	PLGIKVSQKM	DPNELIKL	D	I.N.ANK...			
StshkB	GERTRQLDGA	HVEFLRGVAN	PLGIKVSQKM	DPNELIKL	D	I.N.ANK...			
LeDHS1	GERTRQLDGA	HVEFLRGVAN	PLGIKVSQKM	DPNELIKL	D	I.N.ANK...			
AtDHS1	GERTRQLDGA	HVEFLRGVAN	PLGIKVSQKM	DPNELIKL	D	I.N.ANK...			
StshkA	GERTRQLDGA	HVEFLRGVAN	PLGIKVSQKM	DPNELIKL	D	I.N.ANK...			
LeDHS2	GERTRQLDGA	HVEFLRGVAN	PLGIKVSQKM	DPNELIKL	D	I.N.ANK...			
NtDAHPS	GERTRQLDGA	HVEFLRGVAN	PLGIKVSQKM	DPNELIKL	D	I.N.ANK...			
AtDHS2	GERTRQLDGA	HVEFLRGVAN	PLGIKVSQKM	DPNELIKL	D	I.N.ANK...			
450									
S.rimosus	LTFIVRMGAD	KIRDKLPVL	ERSPPAT-V	AWITDPMHGN	TYEAPVHKT				
StshkB	I.V.....E	NM.V...SH.	RAVRGAGQI	T.VC.....	IK.PCGL...				
LeDHS1	I.V.....E	NM.V...SH.	RAVRGAGQI	T.VC.....	IK.PCGL...				
AtDHS1	I.V.....E	NM.V...SH.	RAVRGAGQI	T.VC.....	IK.PCGL...				
StshkA	I.I.T.....E	NM.V...H.I	RAVRGAGQI	T.VS.....	IK.PCGL...				
LeDHS2	I.I.T.....E	NM.V...H.I	RAVRGAGQI	T.VS.....	IK.PCGL...				
NtDAHPS	I.I.T.....E	NM.V...H.I	RAVRGAGQI	T.VS.....	IK.PCGL...				
AtDHS2	I.V.....E	NM.V...N.I	RAVRGAGQI	T.VS.....	IM.PGGL...				
500									
S.rimosus	RRFDDVLDE	KGFEVH							
StshkB	RRFDDVLDE	KGFEVH							
LeDHS1	RRFDDVLDE	KGFEVH							
AtDHS1	RRFDDVLDE	KGFEVH							
StshkA	RRFDDVLDE	KGFEVH							
LeDHS2	RRFDDVLDE	KGFEVH							
NtDAHPS	RRFDDVLDE	KGFEVH							
AtDHS2	RRFDDVLDE	KGFEVH							
545									
S.lividans	LHQRYETACD	PRLNRSQSLD	LAPLVAEMYR	DQ					
StshkB	GS.H.H...	A...E	S.I...RL	RRRMSQRL					
LeDHS1	GS.H.H...	A...E	S.I...RL	RRRMSQRL					
AtDHS1	SS.H.H...	A...E	S.I...RL	KRRTGSQSV					
StshkA	SS.H.H...	A...E	S.I...RL	KRRTGSQSV					
LeDHS2	SS.H.H...	A...E	S.I...RL	KRRTGSQSV					
NtDAHPS	SS.H.H...	A...E	S.I...RL	KRRTGSQSV					
AtDHS2	SS.H.H...	A...E	S.I...RL	KRRTGSQSV					

Fig. 3. Multiple alignment of the *S. rimosus*, the *N. crassa* and the putative *S. lividans* DAHP synthase sequences with those of plants. The alignment of the *S. rimosus* peptide sequence, the three *N. crassa* peptide sequences and the *S. lividans* DNA sequence encoding part of a putative DAHP synthase with the plant DAHP synthase sequences is shown. All sequences are shown by the single letter amino acid code. The *S. lividans* sequence was determined by L. L. Liaw and others (accession number L13767); the alignment has been improved by allowing for two frameshifts, at the asterisked positions, which are required to keep the reading frame open. The *Arabidopsis* sequences (AtDHS1, AtDHS2) have been taken from Keith *et al.* (1991), the potato sequences (StshkA, StshkB) from Dyer *et al.* (1990) and Zhao & Herrmann (1992), the tobacco sequence (NtDAHPS) from Wang *et al.* (1991) and the tomato sequences (LeDHS1, LeDHS2) from Gorlach *et al.* (1993). The correct endoproteinase Lys-C cleavage site is assumed to be present on the *S. rimosus* enzyme. Dots represent identity with the *N. crassa* or *S. lividans* sequences and conservative amino acid substitutions are underlined. The letter X represents amino acid residues unidentified during the microsequencing. A dash indicates a gap introduced to improve the alignment.

failed to sequence and were presumed to contain the blocked N-terminus. Four peptides yielded sequence; two of these had identical N-terminal amino acid sequences and must be overlapping partial digest products. Altogether 83 residues were obtained from the three non-overlapping peptides (Table 3). The sequences showed similarity to the plant DAHP synthases (Fig. 3). These peptide sequences were unlike any other previously published microbial DAHP synthase sequences.

For the peptide of M_r 19000 (Table 3), the highest degree of identity was found with the *Arabidopsis* (AtDHS1) DAHP synthase in a 30 amino acid overlap (57% identity of amino acid residues). For the peptide of M_r 18000

(Table 3), the highest degree of identity was found with the *Arabidopsis* (AtDHS1), tomato (LeDHS1) and potato (StshkB) DAHP synthases in a 25 amino acid overlap (52% identity of amino acid residues). Finally, the peptide of M_r 13000 (Table 3) exhibited the highest sequence identity with the tomato (LeDHS1) and potato (StshkB) DAHP synthases in a 30 amino acid overlap (43% identity of amino acid residues).

Implications for the classification and evolutionary history of DAHP synthase enzymes

The sequences of the microbial DAHP synthases obtained before the work reported here exhibit a striking level of

Table 3. Amino acid sequences obtained from microsequence analysis

Organism	Sample	Sequence*
<i>S. coelicolor</i>	Intact protein	TVNAKTSPSAGNTD
	25000 M_r peptide	TVNAKTSPSAGNTDDPLQAPS
<i>S. rimosus</i>	10000 M_r peptide	TRRFDDVLDEVKGFFEVH
<i>N. crassa</i>	19000 M_r peptide	TLPPIVLPNEIIRLREHLRDVAQGKAFLQ
	18000 M_r peptide	YQETVDRIQEMLRFMH?IGADQNEK
	13000 M_r peptide	LSTVELFTSHEGLLEEEPLTT?LNAPSQ

* Unidentified amino acids are indicated by '?'.

sequence similarity, suggesting that they are derived from a common ancestral sequence (Pittard, 1987; Ray *et al.*, 1988; Kunzler *et al.*, 1992; Chen *et al.*, 1993; Pereira & Livi, 1993). The DAHP synthase sequences identified in plants similarly display a high degree of similarity but are unrelated to the microbial sequences (Dyer *et al.*, 1990; Keith *et al.*, 1991; Grolach *et al.*, 1993). This information leads to the conclusion that two distinct classes of DAHP synthase have evolved. We define a type I DAHP synthase as having an *E. coli*-like sequence with a monofunctional subunit M_r of around 39000 and a type II DAHP synthase as having a plant-like sequence with a monofunctional subunit M_r of around 54000 (disregarding the chloroplast-targeting region). Our sequence data for the *Streptomyces* and *Neurospora* enzymes indicate that type II DAHP synthases also occur in prokaryotic and eukaryotic microbial species.

This situation is not unprecedented in the shikimate pathway. The third step of the pathway is catalysed by the dehydroquinases, which also fall into two distinct classes, type I and type II (Giles *et al.*, 1985; White *et al.*, 1990; Garbe *et al.*, 1991; Euvrinks *et al.*, 1992; Kleanthous *et al.*, 1992; Hawkins *et al.*, 1993), distinguished by their non-homologous amino acid sequences (Charles *et al.*, 1985; Da Silva *et al.*, 1986; Duncan *et al.*, 1987) and by kinetic, mechanistic, structural and biophysical criteria (Kleanthous *et al.*, 1992; Harris *et al.*, 1993; Gourley *et al.*, 1994).

Variations of the shikimate pathway may exist. Enzymes have been postulated to be capable of catalysing analogous reactions to the orthodox pathway with modified substrates such as amino-DAHP (Kim *et al.*, 1992). Such a modified shikimate pathway may exist in organisms such as *Nocardia* and *Streptomyces* which require the shikimate-pathway-derived precursor 3-amino-5-hydroxybenzoic acid for ansamycin biosynthesis (Kim *et al.*, 1992). These variant pathways may utilize the same shikimate pathway enzymes to synthesize 3-amino-5-hydroxybenzoic acid or they may employ other enzymes. If single enzyme activities fulfil both roles in an individual organism, then some degree of substrate ambiguity would be necessary. It is possible that this requirement for ambiguous substrate specificity may provide an explanation for the presence of a plant-like DAHP synthase in some micro-organisms and not in others.

Prior to this study, no amino acid sequence information was available from any microbial source which indicated that the DAHP synthases from some bacteria and fungi were related to those of plants. Our data establish that plant-like type II DAHP synthases exist in different bacteria and fungi. The sequence conservation between the type II DAHP synthases from such evolutionary distant organisms as *Streptomyces* and plants should assist with the identification of functionally important residues and regions in the enzymes.

ACKNOWLEDGEMENTS

We thank Mr John Greene for technical assistance and Mr Malcolm Horsburgh for help with computer sequence analysis. A major part of this work was supported by the SERC Initiative, Secondary Metabolism of *Streptomyces*, involving Glaxo and SmithKline Beecham.

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Received 1 March 1996; accepted 21 March 1996.