The 3-dehydroquinate synthase activity of the pentafunctional *arom* enzyme complex of *Neurospora crassa* is Zn^{2+} -dependent

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We have demonstrated the co-purification in constant ratio of all five activities of the pentafunctional *arom* enzyme complex from *Neurospora crassa*. Progressive inactivation of the 3-dehydroquinate synthase component of the purified enzyme complex by chelating agents was blocked by the substrate, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate, but not by the cofactor NAD^+ . Full activity was restored at Zn^{2+} concentrations as low as 0.05 nM. Atomic absorption data indicated that the intact enzyme complex contained 1 atom per subunit of tightly bound zinc. The *arom* 3-dehydroquinate synthase had a calculated turnover number of 19s^{-1} , this being within the narrow range of values obtained for the other four activities of the intact multifunctional enzyme. The K_m for 3-deoxy-D-*arabino*-heptulosonate 7-phosphate was 1.4 μM in a phosphate-free buffer; inorganic phosphate was a competitive inhibitor with respect to 3-deoxy-D-*arabino*-heptulosonate 7-phosphate.

3-Dehydroquinate (DHQ) synthase (EC 4.6.1.3) catalyses the second step in the common pathway of aromatic amino acid biosynthesis (Srinivasan *et al.*, 1963; see Scheme 1). In the fungus *Neurospora crassa* the DHQ synthase reaction is the first of five sequential steps in the shikimate pathway that are catalysed by the *arom* enzyme complex (Giles *et al.*, 1967). Purification of the *arom* enzyme complex to electrophoretic homogeneity, in the presence of proteinase inhibitors, revealed it to be a dimer of identical 165 kDa polypeptide chains (Lumsden & Coggins, 1977; Gaertner & Cole, 1977; Lumsden & Coggins, 1978), and superseded earlier claims for a more complex subunit structure (Jacobson *et al.*, 1972; Gaertner, 1972). Genetic and biochemical data indicate that within this 'multifunctional' enzyme complex there are spatially distinct catalytic sites for the DHQ synthase reaction and for the four subsequent biosynthetic steps of the shikimate pathway (Rines *et al.*, 1969; Case & Giles, 1971; Smith, 1980; J. Lumsden & J. R. Coggins, unpublished work). There is evidence

that at least the second plus third component activities, and the fifth activity, are associated with structurally independent and separable catalytic 'domains', derived from non-overlapping segments of the pentafunctional *arom* polypeptide, and hence of the structural gene (Smith & Coggins, 1983; Boocock, 1983). The emerging picture of a multifunctional enzyme built from independent structural units must, however, take account of the claim that interactions between the five catalytic sites endow the *arom* enzyme complex with unique kinetic and regulatory properties (Welch & Gaertner, 1975, 1976).

The *arom* enzyme complexes comprising DHQ synthase and the four subsequent shikimate-pathway activities appear to be restricted to the fungi and the euglenoids (Ahmed & Giles, 1969; Patel & Giles, 1979). The DHQ synthases of plants and bacteria are generally separable from other shikimate-pathway activities (Berlyn & Giles, 1969; Koshiba, 1979). Monofunctional enzymes from *Escherichia coli*, *Bacillus subtilis* and mung bean have been purified to homogeneity and share a number of characteristic properties (Maitra & Sprinson, 1978; Hasan & Nester, 1978; Yamamoto, 1980; Frost *et al.*, 1984). The relationship, if any, of these enzymes to the DHQ synthase component of the *arom* enzyme complex is of particular interest in tracing the evolutionary origin of the pentafunctional *arom* polypeptide.

Abbreviations used: dHepA-7-P, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; DHQ, 3-dehydroquinate; EPSP, 5-enolpyruvoylshikimate 3-phosphate.

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Scheme 1. *Early common pathway of aromatic amino acid biosynthesis*

The *aroM* enzyme complex catalyses the five numbered reactions in the above scheme. It is responsible for the conversion of dHepA-7-P into EPSP. The enzymes involved are: 1, DHQ synthase (EC 4.6.1.3); 2, 3-dehydroquinase (EC 4.2.1.10); 3, shikimate dehydrogenase (EC 1.1.1.25); 4, shikimate kinase (EC 2.7.1.71); 5, EPSP synthase (EC 2.5.1.19), also known as 3-phosphoshikimate 1-carboxyvinyltransferase.

Biochemical approaches to this problem may soon be complemented by nucleotide sequence data, following the successful cloning of the *aro* (aromatic) structural genes from *Aspergillus nidulans*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Kinghorn & Hawkins, 1982; Larimer *et al.*, 1983; Nakanishi & Yamamoto, 1984) and of the DHQ synthase gene and other shikimate-pathway genes from *E. coli* (Duncan & Coggins, 1984; Duncan *et al.*, 1984; Anton & Coggins, 1984).

DHQ synthase catalyses an intramolecular condensation reaction of dHepA-7-P, which yields the C₆ alicyclic ring of DHQ, with the release of P_i (Srinivasan *et al.*, 1963). All known DHQ synthases require catalytic amounts of NAD⁺ for activity. Detailed mechanistic studies on the enzyme from *E. coli* have implicated NAD⁺ as the cofactor in a transient oxidation of the 5-hydroxy group of dHepA-7-P that precedes elimination of

phosphate from the substrate (Maitra & Sprinson, 1978; Rotenberg & Sprinson, 1978). Experiments with synthetic analogues strongly suggest that the true substrate of the enzyme is a closed-ring pyranose anomer of dHepA-7-P (Le Marechal & Azerad, 1976; Maitra & Sprinson, 1978; Le Marechal *et al.*, 1981).

There is evidence that DHQ synthases isolated from *E. coli*, *B. subtilis*, mung beans and sorghum require a bivalent transition-metal cation for catalytic activity (Maitra & Sprinson, 1978; Hasan & Nester, 1978; Yamamoto, 1980; Saijo & Kosuge, 1978). All four enzymes are activated by Co²⁺, and the *B. subtilis* and mung-bean enzymes are also reported to be activated by Mn²⁺ and Cu²⁺ respectively. All four enzymes are inhibited or inactivated by the chelating agent EDTA, and activity is at least partially restored on incubation with excess Co²⁺. It has been reported that under

certain defined conditions the DHQ synthase activity of the *arom* enzyme complex is dependent on the addition of zinc or cobalt salts (Burgoyne *et al.*, 1969). We were therefore surprised to find that the enzyme isolated in our own laboratory (Lumsden & Coggins, 1977) is not stimulated by metal cations under the standard assay conditions and shows full activity in the presence of excess EDTA (G. A. Nimmo & J. M. Lambert, unpublished work). In the present paper we demonstrate that the DHQ synthase component of the *arom* complex is indeed a metalloenzyme, and we report that special precautions are needed to ensure quantitative recovery of this activity during purification.

Materials and methods

Reagents

Bistrispropane {1,3-bis-[2-hydroxy-1,1-bis(hydroxymethyl)ethylaminol]propane}, diphenylthiocarbazone and deoxyribonuclease I were from Sigma Chemical Co., Poole, Dorset, U.K. Nucleotides were from Boehringer Corp., Lewes, East Sussex, U.K. 4-Hydrazinobenzoate was from Fluka A.G. Chemische, Buchs, Switzerland. Ammonium 3-dehydroquinase and 3-dehydroshikimate acid were gifts from Dr. S. Chaudhuri; 5-enolpyruvylshikimate 3-phosphate (EPSP) was prepared by the method of Knowles *et al.* (1970) as modified by Lewendon & Coggins (1983).

All other reagents were purchased from BDH Chemicals, Poole, Dorset, U.K., from Fisons Scientific Apparatus, Loughborough, Leics., U.K., from Aldrich Chemical Co., Gillingham, Dorset, U.K., or from the sources listed in the appropriate references.

Growth of *Neurospora crassa*

Neurospora crassa strain 74-OR23-1A (F.G.S.C. No. 987, obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, CA, U.S.A.) was grown, harvested and stored as freeze-dried powdered cells at -20°C as described by Lumsden & Coggins (1977).

Purification of the *arom* multienzyme complex from *Neurospora crassa*

The *arom* enzyme complex was isolated as described by Lumsden & Coggins (1977) with the modification of Smith & Coggins (1983) in which chromatography on cellulose phosphate was replaced by chromatography on Blue Dextran-Sephadex. In later work small but significant changes were made to the purification procedure in order to increase the recovery of DHQ synthase activity. The purification steps described by Lumsden & Coggins (1977) are listed here together

with the modifications introduced during the present work.

Step 1: extraction and centrifugation. EDTA was omitted from the extraction buffer. Dithiothreitol was replaced by 1.4 mM-2-mercaptoethanol in all buffers for steps 1–5.

Step 2: passage through DEAE-cellulose. This step was performed as described previously.

Step 3: incubation at 37°C with deoxyribonuclease. This step was omitted.

Step 4: fractionation with $(\text{NH}_4)_2\text{SO}_4$. The protein that was precipitated between 40% and 50% saturation with $(\text{NH}_4)_2\text{SO}_4$ was dissolved in 100 mM-Tris/HCl buffer, pH 7.5, containing 1.4 mM-2-mercaptoethanol, 1.2 mM-phenylmethanesulphonyl fluoride and 0.01 mM- ZnSO_4 , and dialysed against 50 mM-Tris/HCl buffer, pH 7.5, containing the same concentrations of 2-mercaptoethanol, phenylmethanesulphonyl fluoride and ZnSO_4 .

Step 5: chromatography on DEAE-cellulose. This step was performed as described by Lumsden & Coggins (1977) except that all buffers contained 0.01 mM- ZnSO_4 , and 1.4 mM-2-mercaptoethanol in place of dithiothreitol.

Step 6: chromatography on Blue Dextran-Sephadex (Smith & Coggins, 1983). The fractions containing *arom* enzyme complex from step 5 (about 100 mg of protein from 100 g of *N. crassa*) were pooled and dialysed overnight against 20 vol. of 50 mM-Tris/HCl buffer, pH 7.5, containing 30 mM-KCl, 1.4 mM-2-mercaptoethanol and 1.2 mM-phenylmethanesulphonyl fluoride, and then applied to a 2 ml column of Blue Dextran-Sephadex previously equilibrated with the same buffer. The column was washed with 6 ml of equilibration buffer and then with 0.1 M-Tris/HCl buffer, pH 7.5, containing 0.5 M-KCl, 1.4 mM-2-mercaptoethanol and 1.2 mM-phenylmethanesulphonyl fluoride until the A_{280} of the eluate was zero. The *arom* enzyme complex was eluted with 0.1 M-Tris/HCl buffer, pH 7.5, containing 1.5 M-KCl, 0.02 mM- ZnSO_4 , 1.4 mM-2-mercaptoethanol and 1.2 mM-phenylmethanesulphonyl fluoride, 1 ml fractions being collected into tubes already containing 25 μl of 50 mM-Tris/HCl buffer, pH 7.5, containing 0.8 mM- ZnSO_4 . Fractions containing *arom* enzyme complex were pooled and dialysed against 50 mM-potassium phosphate buffer, pH 7.0, containing 1.0 mM-benzamidine, 0.4 mM-dithiothreitol, 0.04 mM- ZnSO_4 and 50% (v/v) glycerol, and stored at -20°C . The Blue Dextran-Sephadex column was regenerated by washing with 2–3 column volumes of 50 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-EDTA and 6 M-guanidinium chloride, followed by 10–20 column volumes of 0.1 M-Tris/HCl buffer, pH 7.5, containing 1.9 M-KCl and 5 mM-EDTA, and then by 30 column

volumes of 50mM-Tris/HCl buffer, pH 7.5, containing 30mM-KCl. The column was stored in this Tris buffer/KCl medium containing 0.01% (w/v) NaN_3 .

Preparation of dHepA-7-P

Escherichia coli strain AB2847A (Pittard & Wallace, 1966), an *aro* B⁻ strain that lacks DHQ synthase (kindly provided by Dr. B. J. Bachmann, *E. coli* Genetic Stock Center, New Haven, CT, U.S.A.), was grown at 30°C in 4-litre stirred cultures with vigorous aeration on medium A (Gibson, 1970) lacking glucose. After 16 h the cells were harvested by centrifugation and resuspended in 4 litres of accumulation medium, which contained 1.6% (w/v) glucose and 22 g of KH_2PO_4 /l adjusted to pH 7.1 with 10M-NaOH and final concentrations of 10 μM - FeSO_4 , 10mM- $(\text{NH}_4)_2\text{SO}_4$, 0.5mM- MgSO_4 , 25 μM -L-tryptophan, 50 μM -L-tyrosine, 50 μM -L-phenylalanine, 0.125 μM -*p*-aminobenzoic acid and 0.125 μM -*p*-hydroxybenzoic acid. After a further 16 h at 30°C with vigorous aeration, the cells were removed by centrifugation, and the supernatant, which now contained up to 0.5mM-dHepA-7-P, was diluted 5-fold with distilled water and pumped on to a column (21 cm $\times$ 4.5 cm) of Dowex 1-X8 (Cl^- form; 10–50 mesh). The column was washed with distilled water, and then the sugar phosphates were eluted with 0.6M-HCl. Fractions containing dHepA-7-P were pooled, concentrated by rotary evaporation and diluted to 100 ml, and the pH was adjusted to between 7 and 8 with 15M- NH_3 added dropwise with rapid stirring.

Chromatography on Amberlite 400. The neutralized extract was diluted to a conductivity of 2.5 mS and the solution applied to a 10 cm $\times$ 4.5 cm column of Amberlite CG400 (Cl^- form) (sufficient for 2 mmol of sugar phosphate). The column was washed with water and then with 6mM-HCl until conductivity started to rise steeply (about 8 litres). The column was then developed with a gradient of 6–60mM-HCl (total volume 3 litres). Fractions containing dHepA-7-P (yield 88%) were pooled and concentrated by rotary evaporation.

Precipitation of lithium salt. The dHepA-7-P was precipitated as the lithium salt with ethanol (Trigalo *et al.*, 1975), and freeze-dried. The dry white powder was stored at -20°C .

Determination and analysis of dHepA-7-P

The preparation was analysed on silica thin-layer sheets by chromatography with butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.), and by electrophoresis with pyridine/acetate buffers at pH 3.5 and 6.5 (Bates *et al.*, 1975). Phosphates were located with the FeCl_3 /sulphosalicylic acid stain (Dixon & Sparkes, 1974) or with

the Zinzadze reagent (Christie, 1982), and sugars were located by using the periodate/Schiff method (Christie, 1982); the preparation showed a single sugar phosphate species under all conditions. dHepA-7-P was assayed by using the first three activities of the *arom* enzyme complex, with monitoring of the oxidation of NADPH at 340 nm at 25°C, as described by Nimmo & Coggins (1981). The dry white powder was 31% (by weight) dHepA-7-P; the balance was presumably water of crystallization. The keto group of dHepA-7-P was also assayed by titration with 4-hydrazinobenzoate (Nimmo, 1974) (see the Results section).

Preparation of 3-dehydroquinase from E. coli

3-Dehydroquinase was required as a coupling enzyme in the assay of DHQ synthase. It was partially purified from *E. coli* strain A.T.C.C. 14948 by a simple procedure involving $(\text{NH}_4)_2\text{SO}_4$ fractionation, ion-exchange chromatography on DEAE-cellulose and gel filtration on Sephacryl S-200. The pooled fractions after DEAE-cellulose chromatography had a specific activity of 0.5 unit/mg of protein and were suitable for use as a coupling enzyme. Most assays were, however, performed with 3-dehydroquinase of specific activity 2 units/mg that had been further purified by gel filtration on Sephacryl S-200. The coupling enzyme preparations were dialysed into 50mM-potassium phosphate buffer, pH 7.5, containing 0.4mM-dithiothreitol, 1.0mM-benzamidine and 50% (v/v) glycerol, and stored at -20°C .

Analysis of zinc

Preparations of purified *arom* enzyme complex were dialysed for 9 days against four changes of 10mM-sodium phosphate buffer, pH 7.0, containing 2mM-NaCl that had been extracted with 0.01% (w/v) diphenylthiocarbazone in carbon tetrachloride (Thiers, 1957). All glassware was washed with conc. HNO_3 , then with 0.01% (w/v) diphenylthiocarbazone in carbon tetrachloride and finally with distilled water. Zinc determinations were made on duplicate samples at the zinc resonance line of 213.9 nm (slit 320 μm) in the air/acetylene flame of an IL151 atomic absorption spectrophotometer equipped for background correction with a deuterium continuum lamp. Zinc was undetectable in the diffusate of the final buffer change of the dialysis (limit of detection 0.005 $\mu\text{g}/\text{ml}$). The error in the determination was $\pm 0.003 \mu\text{g}/\text{ml}$ up to 0.05 $\mu\text{g}/\text{ml}$, and $\pm 0.005 \mu\text{g}/\text{ml}$ for values between 0.05 and 0.1 $\mu\text{g}/\text{ml}$.

Enzyme assays

DHQ synthase activity was assayed by coupling the release of DHQ to the 3-dehydroquinase reaction (Welch & Gaertner, 1976). The standard

assay contained final concentrations of 200 μM -dHepA-7-P, 40 μM -NAD⁺ and 60 munits of *E. coli* 3-dehydroquinase/ml in 50 mM-potassium phosphate buffer pH 7.0. For the determination of activity ratios the assay mixtures contained 400 μM -dHepA-7-P, 50 μM -NAD⁺ and 100 mM-potassium phosphate buffer, pH 7.0. For measurements at very low concentrations of substrate the buffer was 12 mM-bis-trispropane/acetate, pH 7.0; all solutions were passed through Millipore filters, and a chart-recorder full-scale deflexion equivalent to 0.02 absorbance unit was used.

The *arom* enzyme complex was normally diluted into 20 mM-potassium phosphate buffer, pH 7.0, containing 0.1 mg of bovine serum albumin/ml, 0.2 mM-dithiothreitol and 20 μM -ZnSO₄. Assays were initiated by adding 0.005 vol. of diluted *arom* enzyme complex; assays of crude extracts were initiated with dHepA-7-P.

3-Dehydroquinase activity was assayed by the method of Mitsuhashi & Davis (1954), taking $\epsilon_{234} = 1.2 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the reaction. The assays contained 100 μM -DHQ, normally in 50 mM-potassium phosphate buffer, pH 7.0, but in 100 mM-potassium phosphate buffer when activity ratios were to be measured.

Shikimate dehydrogenase was routinely assayed by following the reverse reaction at pH 10.6 as described previously (Lumsden & Coggins, 1977). The forward reaction was assayed in medium containing 150 μM -dehydroshikimate, 170 μM -NADPH and 100 mM-potassium phosphate buffer, pH 7.0.

Shikimate kinase was assayed spectrophotometrically at 340 nm by coupling the release of ADP to the pyruvate kinase and lactate dehydrogenase reactions (G. A. Nimmo, unpublished work). The assay medium contained final concentrations of 1 mM-shikimic acid, 2.5 mM-ATP, 1 mM-phosphoenolpyruvate, 100 μM -NADH, 2.5 mM-MgCl₂, 50 mM-KCl and 50 mM-triethanolamine hydrochloride/KOH buffer, pH 7.0, with 3.0 units of pyruvate kinase/ml and 2.5 units of lactate dehydrogenase/ml.

EPSP synthase activity was routinely assayed in the reverse direction in 100 mM-potassium phosphate buffer, pH 7.0, as described previously (Boocock & Coggins, 1983). The forward reaction was assayed spectrophotometrically at 275 nm (Lewendon & Coggins, 1983); the assay medium contained 300 μM -shikimate 3-phosphate, 300 μM -phosphoenolpyruvate, 10 μM -FMN, 20 μM -NADPH, 2.5 mM-MgCl₂, 50 mM-KCl and 50 mM-triethanolamine hydrochloride/KOH buffer, pH 7.0, with 12 munits of chorismate synthase/ml.

All spectrophotometric assays were performed at 25°C in a final volume of 1 ml, with a Gilford/Unicam model 252 spectrophotometer.

One unit of enzyme activity is defined as the amount of enzyme that catalyses the conversion of 1 μmol of substrate/min. Activity ratios of the *arom* enzyme complex at various stages in purification were normally calculated from the results of three or four replicate assays of each activity. Enzyme samples were diluted into 100 mM-potassium phosphate buffer, pH 7.0, before assay. Assays of partially purified enzyme fractions were initiated by the addition of appropriate pathway intermediates, and any blank rates were subtracted. A chart-recorder full-scale deflexion equivalent to 0.1 absorbance unit was used for all determinations of activity ratios, and an electronic 'back-off' facility was essential for many of the assays. All assays had good linearity in the range of absorbance changes employed.

Analytical methods

Conductivity measurements were made with a Radiometer type CDM2e conductivity meter (Radiometer, Copenhagen, Denmark).

The concentration of purified *arom* enzyme complex was measured at 280 nm, assuming $A_{1\text{cm}}^{1\%}$ 11.0 (Lumsden & Coggins, 1978). Other protein determinations were made by the method of Bradford (1976), with bovine serum albumin as the standard.

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was by the method of Laemmli (1970).

Results

Purification of the *arom* enzyme complex

Comparison of several electrophoretically homogeneous samples of the *arom* enzyme complex prepared by the method of Lumsden & Coggins (1977), as modified by Smith & Coggins (1983), revealed that the specific DHQ synthase activity varied in the range of 10–40% of the specific 3-dehydroquinase activity. There were also wide variations in the specific EPSP synthase activity (particularly after prolonged storage), but the specific activities of 3-dehydroquinase, shikimate dehydrogenase and shikimate kinase were essentially invariant, in accord with data presented earlier (Lumsden & Coggins, 1977). Further investigation established that the ratio of DHQ synthase to 3-dehydroquinase activity in crude mycelial extracts was close to 1:1, and that serious losses of DHQ synthase activity were sustained in the final two chromatographic steps of the purification procedure, particularly during chromatography on Blue Dextran-Sepharose. Preliminary experiments revealed that the residual DHQ synthase activity of the purified *arom* enzyme complex was progressively destroyed on incuba-

tion with EDTA, but could be recovered on incubation with excess Zn^{2+} ion, or in metal-ion 'buffers' at nanomolar concentrations of free Zn^{2+} ion. The selective losses of DHQ synthase activity sustained during chromatography were not reversed by incubating the enzyme with zinc salts. These observations led us to a re-examination of the original purification procedures.

Modifications to the established purification scheme were assessed by monitoring the relative activities of all five component enzymes of the *arom* enzyme complex at each stage. We were concerned to avoid strong chelating agents and sources of adventitious metal ions, which we suspect may 'poison' the zinc-binding site of the enzyme. EDTA was omitted from the extraction buffer, and 2-mercaptoethanol replaced dithiothreitol as the protective thiol reducing agent, since the latter is a relatively strong metal-ion-chelating agent, with pK_a for Zn^{2+} of 10.3 (Cornell & Crivaro, 1972). Oxidative damage to the sensitive EPSP synthase component of the *arom* enzyme complex only presented serious problems during storage, and dithiothreitol was therefore retained in the final dialysis buffer. Selective losses of DHQ synthase activity during chromatography were avoided by supplementing appropriate buffers with $10\text{ }\mu\text{M}$ - ZnSO_4 . The capacity of the Blue Dextran-Sephadex affinity matrix was drastically diminished when it was equilibrated with buffers containing Zn^{2+} , presumably because the Zn^{2+} ions interact with the immobilized dye and block the enzyme-binding sites. It was therefore necessary to dialyse the enzyme pool from the ion-exchange step before proceeding to affinity chromatography. Details of the final purification protocol are given in the Materials and methods section.

In a full-scale experiment to evaluate the modified purification protocol, electrophoretically homogeneous *arom* enzyme complex was obtained in 43% overall yield after a 730-fold purification with respect to the 3-dehydroquinase activity (Table 1). Relative activities of the five component

enzymes during purification were recorded as activity ratios, taking the robust 3-dehydroquinase activity as the reference point (Table 2). The final two chromatographic steps achieved a 70-fold co-purification of the five activities of the *arom* enzyme complex with the maintenance of essentially constant activity ratios. There was no evidence of any substantial change in the activity ratios in the earlier stages of purification, although shikimate kinase activity in crude extracts could not be assayed, and the DHQ synthase assays were technically difficult. The purified enzyme did not differ significantly from the material obtained by the earlier method in its specific 3-dehydroquinase activity, nor in the ratios of its 3-dehydroquinase, shikimate dehydrogenase and shikimate kinase activities. The final DHQ synthase/3-dehydroquinase activity ratio was 2.5-fold higher than previously seen, confirming the quantitative recovery of this activity at each stage of purification. In independent experiments with the modified purification procedure, the final specific activities and activity ratios were very similar, and all five activities were stable on prolonged storage (more than 1 year) at -20°C . Taken together, these results constitute good evidence that the *arom* enzyme complex has now been isolated with its full complement of enzyme activities.

The shikimate dehydrogenase and EPSP synthase activities of the *arom* enzyme complex were routinely assayed in the 'reverse' direction. Activity ratios were therefore redetermined for the five 'forward' biosynthetic reactions at pH 7.0, with a fresh sample of the intact purified enzyme (Table 3). The calculated turnover number of the DHQ synthase component is 19.0 s^{-1} . This is very similar to the turnover number for the monofunctional *E. coli* DHQ synthase of 24 s^{-1} obtained from the data of Maitra & Sprinson (1978).

Isolation of dHepA-7-P

We have developed a method for the large-scale purification of dHepA-7-P from culture supernatants of an *E. coli* mutant lacking DHQ synthase

Table 1. *Purification of the arom multienzyme complex from Neurospora crassa*
Starting material was 100g of freeze-dried mycelia. For full experimental details see the Materials and methods section. The protein concentration in the final pool was estimated from A_{280} measurements (Lumsden & Coggins, 1978); other protein estimations were by the method of Bradford (1976).

Purification step	Protein (mg)	3-Dehydroquinase activity (units)	Yield (%)	Specific activity (units/mg)	Purification (fold)
1. Extract	7020	67.7	100	0.0096	1.0
2. First passage through DEAE-cellulose	3820	68.7	101	0.018	1.9
3. 40–50%-satn. $(\text{NH}_4)_2\text{SO}_4$	654	64.3	95	0.098	10.1
4. DEAE-cellulose chromatography	118	43.0	64	0.36	37.8
5. Blue Dextran-Sephadex chromatography	4.13	29.0	43	7.04	730

Table 2. Activity ratios during the purification of the *arom* enzyme complex. The arrows indicate whether the activities were assayed in the forward (→) or reverse (←) reactions. Activity ratios were calculated from quadruplicate assays except those in parentheses. Further experimental details are given in the Materials and methods section.

Purification step	Purification (fold)	Relative enzyme activity				
		DHQ synthase (→)	3-Dehydroquinase (→)	Shikimate Dehydrogenase (←)	Shikimate kinase (→)	EPSP synthase (←)
1. Extract						(49)
2. First DEAE-cellulose step	1.0	-	100	248	-	(56)
3. 40–50%-satn. (NH ₄) ₂ SO ₄	1.9	(94)	100	259	-	75
4. DEAE-cellulose chromatography	10.1	82	100	265	84	76
5. Blue Dextran–Sephadex chromatography	37.8	97	100	263	82	69
	730	94	100	270	88	

activity. To estimate the proportion of dHepA-7-*P* present in solution as an open-chain ketose, the sugar phosphate was titrated spectrophotometrically with 4-hydrazinobenzoate. This method had previously been used to show that a furanose ring structure predominates in solutions of fructose 1,6-bisphosphate (Nimmo, 1974). The reaction of dHepA-7-*P* with 4-hydrazinobenzoate proceeds with a small burst, corresponding to only 0.3% of the final absorbance deflexion, and then continues with first-order kinetics with respect to dHepA-7-*P* and an estimated relaxation time of 3000 min. Our interpretation of this result is that at equilibrium over 99% of the sugar phosphate is present in a closed-ring pyranose form, and that a slow ring-opening step precedes reaction of the free ketose with 4-hydrazinobenzoate. This interpretation is consistent with evidence that the protons at the C-3 position of dHepA-7-*P* do not exchange rapidly with the solvent (Floss *et al.*, 1972). In addition, we have shown that quantitative conversion of dHepA-7-*P* into DHQ by the *arom* enzyme complex can be completed within 30s, which implies that a 4-hydrazinobenzoate-resistant species (presumably an anomer of the pyranose form of dHepA-7-*P*) is the true substrate of the enzyme, as predicted from the studies with substrate analogues (Le Marechal & Azerad, 1976).

Kinetic properties of DHQ synthase

DHQ synthase was routinely assayed by coupling DHQ release to the 3-dehydroquinase reaction, but the intrinsic 3-dehydroquinase activity of the *arom* enzyme complex was wholly inadequate for this purpose. Satisfactory coupled assays (McClure, 1969) were obtained by adding appropriate amounts of *E. coli* 3-dehydroquinase. This indicated that there is no compulsory transfer of DHQ between the DHQ synthase and the 3-dehydroquinase components of the multifunctional enzyme.

The phosphate buffers used for routine DHQ synthase assays are unsuitable for use at limiting substrate concentrations because of product inhibition by P_i. Other buffers that are transparent at 234nm are unsuitable for a different reason; the *arom*-complex and *E. coli* 3-dehydroquinases are inhibited by most common anions except phosphate and pyrophosphate, but these latter anions both strongly inhibit DHQ synthase. Chloride is a particularly good competitive inhibitor of the two 3-dehydroquinases (*K_i* values of 17mM and 12.5mM for the *E. coli* and *arom*-complex activities respectively), and prohibitively large amounts of coupling enzyme were therefore required for assays in chloride-based buffers. Acetate is a weaker competitive inhibitor (*K_i* of 102mM for the *E. coli* enzyme), and the assay

Table 3. Activity ratios and turnover numbers for the reactions of the purified *arom* enzyme complex. Activity ratios for the forward reactions were determined as described in the Materials and methods section at pH 7.0 at 25°C. Turnover numbers were calculated on the basis of a specific 3-dehydroquinase activity of 7.04 units/mg of protein (Table 1) and assume one active site per 165 kDa subunit. Note that the specific activities previously reported were for shikimate dehydrogenase assayed in the reverse reaction at pH 10.6 at 37°C (Lumsden & Coggins, 1977). Welch & Gaertner (1976) do not report specific activities for the forward reactions.

Enzyme	Relative activity	Turnover number (s ⁻¹)	Relative activity reported by Welch & Gaertner (1976)
DHQ synthase	98	19.0	5 (10*)
3-Dehydroquinase	100	19.4	100
Shikimate dehydrogenase	264	51.2	20
Shikimate kinase	94	18.3	10
EPSP	174	33.6	25

*'Activated' value in the presence of 1 mM-dHepA-7-P.

buffer chosen for kinetic studies at limiting substrate concentrations was Bistrispropane/acetate, pH 7.0.

DHQ synthase gave linear double-reciprocal plots of initial velocity versus [dHepA-7-P] when assayed in Bistrispropane buffers. A K_m for dHepA-7-P of 1.4 μ M and V_{max} of 6.9 units/mg were obtained at 40 μ M-NAD⁺ in 12 mM-Bistrispropane/acetate buffer, pH 7.0. The buffer concentration had little effect on the kinetic parameters in the range 10–60 mM. Phosphate acts as a parabolic competitive inhibitor with respect to dHepA-7-P when the synthase is assayed in potassium phosphate buffer, pH 7.0 (Fig. 1). A K_m for dHepA-7-P of 2 μ M was obtained under these conditions by extrapolating the replot of primary slopes to zero phosphate concentration (Fig. 1). The DHQ synthase had an absolute requirement for catalytic amounts of NAD⁺ and was strongly inhibited by NADH. At 8 μ M-dHepA-7-P the apparent K_m for NAD⁺ was less than 0.2 μ M.

Inactivation of DHQ synthase by chelating agents

No inhibition of DHQ synthase activity was observed when EDTA was added to complete assay mixtures containing *arom* enzyme complex, dHepA-7-P and NAD⁺. However, in the presence of substrates DHQ synthase was progressively inactivated by the chelating agents EDTA, 1,10-phenanthroline and dithiothreitol (Fig. 2). Pseudo-first-order decay of DHQ synthase activity was observed at fixed concentrations of chelating agents, although deviations from linearity were noted with EDTA and dithiothreitol after 50% inactivation. Plots of $\log(1/t_1)$ versus $\log$ [chelator] were linear for inactivation by EDTA and dithiothreitol, with slopes of 0.23 and 1.0 respectively (Fig. 2). The first-order inactivation with respect to [dithiothreitol] indicates that the metal ion is actively removed from the enzyme by this chelat-

Fig. 1. Inhibition of DHQ synthase by phosphate. Double-reciprocal plot of initial velocity (v) against [dHepA-7-P] at a series of concentrations of potassium phosphate: $\circ$, 6 mM; $\blacksquare$, 11 mM; $\triangle$, 26 mM; $\bullet$, 51 mM; $\square$, 101 mM. Inset: variation of primary slope (arbitrary units) as a function of [phosphate].

ing agent. The kinetics of inactivation were also close to first-order with respect to [1,10-phenanthroline] at concentrations below 0.5 μ M. In the absence of substrates, Bistrispropane (50 mM) also inactivated DHQ synthase, probably because of its ability to bind metal ions, although no inhibition was observed with assays in 12 mM-Bistrispropane buffer that were initiated by adding enzyme.

dHepA-7-P and NAD⁺ were tested separately for their ability to protect the synthase against inactivation by 0.5 mM-EDTA at 0°C. dHepA-7-P gave good protection, whereas NAD⁺ was ineffec-

Fig. 2. Inactivation of DHQ synthase by chelating agents

Studies of the inactivation of DHQ synthase by chelating agents were made on *arom* enzyme complex that had been dialysed into buffers lacking bivalent-metal ions. (a) Semi-logarithmic plot of the inactivation of DHQ synthase by different chelating agents as a function of time. The reaction mixtures, incubated at 0°C, contained 50 mM-potassium phosphate buffer, pH 7, 0.05 μM *arom* enzyme complex and various concentrations of chelating agents: □, 3 mM-dithiothreitol; ●, 0.1 mM-EDTA; Δ, 0.6 μM-1,10-phenanthroline. Samples were removed at intervals and diluted 100-fold into the standard DHQ synthase assay mixture. (b) Determination of the order of the inactivation of DHQ synthase by EDTA and dithiothreitol with respect to [chelator]. The half-time of inactivation (*t*_{1/2}, in minutes) for each [chelator] value (mM) was calculated from the slopes of plots such as those in (a). These data were replotted as log(1/*t*_{1/2}) against log[inhibitor]: ●, EDTA; □, dithiothreitol.

tive (Fig. 3). The degree of protection given by 45 μM-dHepA-7-P in 20 mM-potassium phosphate buffer, pH 7 (Fig. 3a), was similar to the degree of protection afforded by only 5 μM-dHepA-7-P in 50 mM-Tris/HCl buffer, pH 7.5 (Fig. 3b). This observation is consistent with the kinetic evidence that phosphate competes with dHepA-7-P for its binding site (Fig. 1).

The activity of native *arom*-complex DHQ synthase was not affected by 1 mM-Mg²⁺, -Ca²⁺, -Mn²⁺ or -Co²⁺, but was partially inhibited by 1 mM-Zn²⁺. The enzyme was very sensitive to inactivation by Fe²⁺, Ni²⁺ and Cu²⁺. Inactivation of DHQ synthase by EDTA was only blocked by metal ions such as Ca²⁺, Mn²⁺, Co²⁺ or Zn²⁺ when the metal ion was in slight excess over the chelating agent.

Reconstitution of DHQ synthase activity after EDTA treatment

The reconstitution of DHQ synthase activity after treatment of the *arom* enzyme complex with EDTA was studied with metal-ion buffers constructed according to the methods of Perrin & Dempsey (1974). Free Zn²⁺ ion concentrations as low as 0.01 nM (pZn 11) are readily defined

in solutions where [Ca²⁺] (or [Mg²⁺]) > [EDTA] > [Zn²⁺], by virtue of competition between Ca²⁺ (or Mg²⁺) and Zn²⁺ for available EDTA. The EDTA-treated enzyme was not significantly reactivated in the control assay mixtures containing Ca²⁺ (or Mg²⁺) and EDTA. In contrast, activity was rapidly recovered on dilution into complete assay mixtures at pZn 8.8 (Fig. 4). Reversal of EDTA inactivation was still detectable at pZn 10.7, but was not seen in assay mixtures containing EDTA (1 mM) and Zn²⁺ (0.5 mM), with no competing bivalent cations (pZn 13.9). These results indicated that in the presence of dHepA-7-P and NAD⁺ the apparent dissociation constant for Zn²⁺ is less than 0.01 nM and that the reassociation reaction is very rapid with *k*_{on} ≥ 10⁷ M⁻¹·s⁻¹.

Slight reconstitution of the latent DHQ synthase activity was observed with Co²⁺ (pCo 8.8), but not with Mn²⁺ (pMn 7.8) (Fig. 4). When Zn²⁺ (final pZn 8.8), was added to these assay mixtures after 60 min, no further reactivation was observed during an additional 20 min period. These observations suggest that Co²⁺ and Mn²⁺ can occupy the Zn²⁺ site and are not readily displaced by Zn²⁺ to reconstitute full enzyme activity.

Fig. 3. Protection of DHQ synthase from EDTA inactivation by dHepA-7-P

(a) Semi-logarithmic plot of the inactivation of DHQ synthase by EDTA as a function of time. The reaction mixtures, incubated at 0°C, contained 20 mM-potassium phosphate buffer, pH 7, 0.5 mM-EDTA, 0.05 μ M *arom* enzyme complex and various concentrations of dHepA-7-P: $\bullet$, 0 μ M; $\square$, 7 μ M; $\blacktriangle$, 45 μ M. Samples were removed at intervals and diluted 100-fold into the standard assay mixture for measuring DHQ synthase activity. (b) The plot of an experiment similar to that in (a) except that 50 mM-Tris/HCl buffer, pH 7.5, replaced 20 mM-potassium phosphate buffer, pH 7. The concentrations of dHepA-7-P were as follows: $\bullet$, 0 μ M; $\square$, 2 μ M; $\blacktriangle$, 5 μ M.

Zinc content of the purified enzyme complex

A number of preparations of electrophoretically homogeneous *arom* enzyme complex were analysed for zinc after exhaustive dialysis against a metal-ion-free buffer. The DHQ synthase/3-dehydroquinase activity ratios were not severely depressed after dialysis, in line with the evidence that the metal cofactor dissociates very slowly in solution. There was a strong correlation between the final DHQ synthase/3-dehydroquinase activity ratio

Fig. 4. Restoration of DHQ synthase activity by bivalent-metal ions after inactivation with EDTA

The *arom* enzyme complex (2.8 μ M) was diluted 40-fold into 50 mM-Tris/acetate buffer, pH 7.5, containing 25 mM-EDTA, and incubated at 0°C until the DHQ synthase activity was abolished. Samples were then diluted a further 100-fold into assay cuvettes (1 ml) containing (final concentrations) 20 mM-Tris/acetate buffer, pH 7.5, 0.135 mM-dHepA-7-P, 0.04 mM-NAD⁺, 0.06 unit of coupling enzyme/ml, 10 mM-CaCl₂ (or 10 mM-MgCl₂ in the case of pZn 10.7), 4 mM-EDTA and various concentrations of bivalent-metal ions to give the final buffered concentrations of free metal ion indicated in the Figure (pZn is defined as $-\log[\text{Zn}^{2+}]$). The compositions of the bivalent-metal ion buffers with respect to Ca²⁺ (or Mg²⁺), EDTA and the transition-metal ion were calculated as described by Perrin & Dempsey (1974), by using the data listed therein. DHQ synthase activity was measured at 25°C by continuous monitoring of the absorbance change at 234 nm. 3-Dehydroquinase activity, which was unaffected by EDTA, was also measured by diluting samples from the 25 mM-EDTA incubation mixture 200-fold into the standard assay mixture. The results are expressed as the ratio of DHQ synthase activity to 3-dehydroquinase activity (%) plotted against the time from dilution of the EDTA-treated *arom* enzyme complex into the metal-ion-buffered assay mixture: $\bullet$, pZn 8.8; $\blacktriangle$, pZn 9.6; $\blacksquare$, pZn 10.0; $\circ$, pZn 10.7; $\triangle$, pCo 8.8; $\square$, pMn, 7.8.

(expressed as a fraction of the maximal ratio seen in the intact enzyme) and the zinc content, calculated as mol of Zn per mol of *arom*-complex subunits (Table 4). The specific DHQ synthase activity thus appears to be directly related to the zinc content of the enzyme (irrespective of whether or not chromatography buffers were supplemented with zinc salts during enzyme purification). On this basis it can be calculated that the 'maximal' DHQ synthase/3-dehydroquinase activity ratio of 94:100 recorded for preparation 3 immediately after purification (Table 2) would correspond to a zinc content of 0.93 atom/subunit. Since we have

Table 4. Comparison of zinc content and DHQ synthase activity of three preparations of the *arom* enzyme complex

Enzyme samples were exhaustively dialysed against a metal-ion-free buffer (see the Materials and methods section) and the specific 3-dehydroquinase activities and DHQ synthase/3-dehydroquinase activity ratios were then redetermined (protein concentrations were estimated from A_{280} measurements; Lumsden & Coggins, 1978). The total zinc concentration in each sample was measured by atomic absorption spectroscopy. The zinc content, expressed as mol of Zn/mol of *arom*-complex subunit, is compared with the final DHQ synthase/3-dehydroquinase activity ratio, expressed as a fraction of the maximum ratio of 0.94 recorded for preparation 3 before exhaustive dialysis against metal-ion-free buffer (see Table 2). The subunit size of the enzyme complex is taken as 165 kDa (Lumsden & Coggins, 1977). Samples 1 and 2 were purified by the method of Smith & Coggins (1983) with no Zn^{2+} supplements in the chromatography buffers, whereas sample 3 was purified as shown in Tables 1 and 2. In this Table E1 refers to DHQ synthase and E2 to 3-dehydroquinase.

Preparation	E2 specific activity after dialysis (units/mg)	E1/E2 activity ratio after dialysis	[<i>arom</i> -complex subunit] (μ M)	Concn. of Zn^{2+} (μ M)	Zinc content (mol of Zn/mol of <i>arom</i> -complex subunit)	Final E1/E2 ratio (after dialysis)	
						Maximum	E1/E2 ratio observed
1	6.32	0.14	2.85	0.58	0.20	0.15	
2	4.79	0.41	2.36	1.18	0.50	0.44	
3	6.49	0.68	2.36	1.57	0.67	0.71	
Dialysis buffer				≤ 0.08			

presented evidence that preparation 3 carried a full complement of enzyme activities, we can conclude that a single zinc atom per *arom*-complex subunit is required for DHQ synthase catalysis.

Discussion

The problems encountered in isolating the *arom* enzyme complex in a proteolytically undamaged form have been discussed previously (Lumsden & Coggins, 1977). In the present paper we have shown that the DHQ synthase component of the complex is selectively inactivated during chromatography, presumably through loss of the zinc cofactor. The instability of DHQ synthase during chromatography is reminiscent of that reported for another zinc-dependent enzyme, phosphomannose isomerase from yeast (Gracy & Noltmann, 1968). After making modifications to our earlier protocol we have been able to demonstrate the co-purification of all five component activities of the *arom* enzyme complex with the maintenance of constant activity ratios. The new procedure yields enzyme that is homogeneous with respect to polypeptide structure and catalytic activities.

The intact *arom* multifunctional enzyme obtained in the present study has catalytic properties very different from those previously reported (Welch & Gaertner, 1976). Turnover numbers for the five component enzymes assayed in the 'forward' biosynthetic reactions all fall in the narrow range from 19 s⁻¹ to 52 s⁻¹ (Table 3). The functional significance of this striking convergence of turnover numbers is not clear, although a similar pattern has been observed with the tetrafunctional tryptophan-biosynthetic enzyme complex of *Euglena gracilis* (Hankins & Mills, 1977). The first and fifth components of the *arom* multifunctional enzyme have very low limiting K_m values for the pathway intermediates, 1.4 μ M for dHepA-7-*P* and 0.36 μ M for shikimate 3-phosphate (Boocock & Coggins, 1983). These values are substantially lower than K_m values of 60 μ M for dHepA-7-*P* and 100 μ M for shikimate 3-phosphate previously estimated under severe product inhibition in a phosphate-based assay buffer (Welch & Gaertner, 1976). It should be made clear that the *arom* enzyme complex used in the 'substrate-channeling' experiments of Welch & Gaertner (1975) was not only proteolytically degraded, as has previously been recognized (Gaertner, 1978), but was also seriously deficient in DHQ synthase activity, as evidenced by the low DHQ synthase/3-dehydroquinase activity ratio (Welch & Gaertner, 1976; see Table 3). For these reasons we contend that it would be premature to draw any conclusions about the occurrence of catalytic coupling within the *arom* multifunctional enzyme (Gaertner, 1978).

until the overall kinetic properties of the intact complex have been thoroughly investigated. No conclusive evidence of catalytic interactions between the component enzymes, nor of 'co-ordinate activation' (Welch & Gaertner, 1976) has been obtained in our laboratory (G. A. Nimmo, J. M. Lambert & M. R. Boocock, unpublished work).

The DHQ synthase activity of the *arom* enzyme complex resembles the monofunctional enzymes of a number of bacteria and plants in its requirement for NAD^+ and its sensitivity to chelating agents, but it differs in requiring Zn^{2+} rather than Co^{2+} for catalytic activity. Zn^{2+} apparently cannot effectively substitute for Co^{2+} in the synthases from *E. coli*, *B. subtilis* and sorghum (Maitra & Sprinson, 1978; Hasan & Nester, 1978; Saijo & Kosuge, 1978).

The identification of Zn^{2+} as the most likely physiological cofactor of the *arom*-complex DHQ synthase rests on several lines of evidence. Firstly, the kinetics of DHQ synthase reconstitution in Zn^{2+} -ion buffers are so rapid that interference from adventitious metal ions can be discounted. Secondly, we have shown that the zinc content of the enzyme purified without Zn^{2+} supplements in the chromatography buffers is correlated with the residual DHQ synthase activity. Finally, we have been unable to identify any metal cation other than Zn^{2+} that significantly activates the EDTA-treated enzyme, and we have shown that the enzyme purified in Zn^{2+} -supplemented chromatography buffers has a DHQ synthase/3-dehydroquinase activity ratio very similar to that of crude mycelial extracts. One possible explanation of the protection afforded by dHepA-7-P against inactivation by chelating agents is that the Zn^{2+} ion is bound in or near the DHQ synthase active site. If this is the case then it may turn out that the electrophilic properties of the Zn^{2+} ion are important in the catalytic cycle.

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