Efficient independent activity of a monomeric, monofunctional dehydroquinate synthase derived from the N-terminus of the pentafunctional AROM protein of *Aspergillus nidulans*

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The dehydroquinate synthase (DHQ synthase) functional domain from the pentafunctional AROM protein of *Aspergillus nidulans* has previously been overproduced in *Escherichia coli* [van den Hombergh, Moore, Charles and Hawkins (1992) Biochem J. **284**, 861–867]. We now report the purification of this domain to homogeneity and subsequent characterization. The monofunctional DHQ synthase was found to retain efficient catalytic activity when compared with the intact pentafunctional AROM protein of *Neurospora crassa* [Lambert, Boocock and Coggins (1985) Biochem J. **226**, 817–829]. The apparent $k_{cat.}$ was estimated to be 8 s^{-1}, and the apparent K_m values for NAD^+ and 3-deoxy-D-*arabino*-heptulosonate phosphate (DAHP) were 3 μM and 2.2 μM respectively. These values are similar to those reported for the intact *N. crassa* enzyme, except that the apparent K_m for NAD^+ reported here is 15-fold higher. The monofunctional DHQ synthase domain is inactivated by treatment with chelating agents in the absence of substrates and is re-activated by the addition of metal ions; among those tested, Zn^{2+} gave the highest $k_{cat.}/K_m$ value. The enzyme is inactivated by diethyl pyrocarbonate; both the substrate, DAHP, and the product phosphate protected against inactivation. Size-exclusion chromatography suggested an M_r of 43000 for the monofunctional domain, indicating that it is monomeric and compactly folded. The c.d. spectrum confirmed that the domain has a compact globular conformation; the near-u.v. c.d of zinc- and cobalt-reactivated domains were superimposable.

INTRODUCTION

7-Phospho-3-deoxy-D-*arabino*-heptulosonate phosphate phosphate-lyase (cyclizing) [3-dehydroquinate (DHQ) synthase, EC 4.6.1.3] catalyses the conversion of 3-deoxy-D-*arabino*-heptulosonate phosphate (DAHP) into 3-dehydroquinate (DHQ). This is the second of the seven reactions in the common pathway of aromatic-amino-acid biosynthesis, the prechorismate or 'shikimate' pathway. The shikimate pathway is present in bacteria, fungi and plants, and its absence in vertebrates has led to investigations into the suitability of this pathway as a target for antibiotics and herbicides [1].

In yeast [2], filamentous fungi [3,4] and flagellates [5] the five central steps of the pathway are catalysed by the pentafunctional AROM protein. The gene encoding the AROM protein (*aromA* in *Aspergillus nidulans*) appears to have arisen by the fusion of five genes which encode monofunctional polypeptides which carry the individual activities [6,7,8]. *Escherichia coli* possesses five separate monofunctional enzymes, as do the other bacteria examined [9]. Within the multifunctional AROM protein the five reactions are catalysed by spatially distinct active sites which are located within individual functional domains and occupy contiguous regions of the polypeptide chain [8,10]. It is thus predicted that individual activities of the AROM protein can be expressed individually by production of the discrete functional domain in isolation from the remainder of the AROM polypeptide. Recently we have overproduced the *A. nidulans* DHQ synthase [11], and the 3-dehydroquinate hydrolyase (DHQase) [12] domains in *E. coli* as active monofunctional polypeptides, and DHQ synthase/phosphoenolpyruvate:3-phosphoshikimate 5-*O*-(1-carboxyvinyl)transferase (EC 2.5.1.19), and DHQase/shikimate dehydrogenase as active bifunctional polypeptides in *E. coli* and *A. nidulans* respectively [13]. There is evidence that both the activator and repressor proteins of the quinate utilization pathway (which shares the metabolites DHQ and DHS) in the filamentous fungi *N. crassa* and *A. nidulans* have sequence similarity to the pentafunctional AROM protein. The activator appears to be related to the two N-terminal domains and the repressor to the three C-terminal domains. The suggested mechanism is that the gene encoding the pentafunctional AROM protein is duplicated and split in two before a DNA-binding domain is inserted near the N-terminus of what becomes the activator protein [14]. The binding capabilities of the three C-terminal enzymes of the AROM protein appear to have been converted into the recognition of inducers [15,16].

The enzymic conversion of DAHP into DHQ was first described by Srnivassan et al. [17]. Monofunctional DHQ synthases have been purified to homogeneity from *E. coli* [18], sorghum (*Sorghum* sp.) [19] and mung bean (*Phaseolus aureus*) [20] and, in addition, the DHQ synthase function of the pentafunctional AROM protein of *N. crassa* has been studied [21]. A common feature shared between these four enzymes is that bivalent metal ions and NAD^+ are required for activity. Where examined, the purified enzymes have been shown to contain stoichiometric amounts of metal ions, Co^{2+} for the *E. coli* enzyme [22] and Zn^{2+} [21] for the *N. crassa* enzyme, while the mung-bean enzyme preparation contained substoichiometric amounts of copper [20]. It has been suggested that zinc may be the functional metal for the *E. coli* enzyme *in vivo*, on the basis of its greater bioavailability [22].

Abbreviations used: DHQ synthase, dehydroquinate synthase [7-phospho-3-deoxy-D-*arabino*-heptulosonate 7-phosphate phosphate-lyase (cyclizing) (EC 4.6.1.3)]; DAHP, 3-deoxy-D-*arabino*-heptulosonate phosphate; DHQ, 3-dehydroquinate; DHQase, 3-dehydroquinate dehydratase (EC 4.2.1.10); DEPC, diethyl pyrocarbonate; DHS, dehydroshikimate; DTT, dithiothreitol; GdnCl, guanidinium chloride.

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The mechanism of DHQ synthase has been intensively studied, using the *E. coli* enzyme as a model ([22–24] and references therein). An unusual property of DHQ synthase was that NAD^+ was required for activity, but was not consumed in the reaction. The reaction involves oxidation, β-elimination, reduction and an intramolecular aldol condensation. Although recent studies have shown that certain steps are likely to be spontaneous and that an oxygen of the phosphate group acts as a base in a deprotonation step [25], the mechanism currently requires the enzyme to bind substrate, metal, NAD^+ and the proposed transition states. This clearly places considerable structural demands on the protein.

In the present paper we report the purification to homogeneity of the DHQ synthase domain of the AROM protein of the filamentous fungus *A. nidulans*. The kinetic properties of the domain are compared with those of the corresponding activity of the intact pentafunctional enzyme of the related species *N. crassa*. Size-exclusion chromatography and c.d. studies indicate that the domain is monomeric and has a compact globular structure. Some mechanistic aspects of the DHQ synthase reaction were also investigated, including the effect of pH, the kinetics of inactivation by chelating agents and inactivation by the histidine-modifying reagent diethyl pyrocarbonate (DEPC).

EXPERIMENTAL

Materials

DAHP was isolated from the growth medium of the auxotrophic *E. coli aroB*$^-$ strain AB2847A [26] as described in [21] and was kindly given by David Gourley (University of Glasgow). DHQ was prepared from quinic acid by the method of Grewe and Handler [27]. *Salmonella typhi* DHQase was purified from an overproducing strain of *E.coli* as previously described [28]. NAD^+ (grade III-C) was obtained from Sigma. All other reagents and chemicals were obtained from Sigma or BDH Chemicals (both of Poole, Dorset, U.K.) and were of AnalaR grade.

Nucleotide sequencing of the PCR-derived DHQ synthase clone

The DNA sequence of the DHQ synthase-encoding region of pTR51 [13,11] was determined by the dideoxy chain-termination method [29] after subcloning appropriate fragments into M13mp18 and M13mp19 [30]. This was to verify that no adventitious mutations were introduced during the PCR-mediated amplification of the DHQ synthase-encoding sequences. The preparation of oligonucleotides and the sequencing were performed as previously described [29,31,32].

Isolation and purification of a monofunctional *A. nidulans* DHQ synthase from an overproducing strain of *E. coli*

E. coli strain GLW38 (aroB$^-$; Glasgow culture collection) harbouring plasmid pTR51 was grown at 37 °C to an attenuance (D_{500}) of 0.2 in 10 × 500 ml of Luria broth in 2-litre baffled flasks. The production of DHQ synthase was induced by the addition of 200 μg of isopropyl β-D-galactoside/ml and incubation was continued for 4 h at 37 °C with agitation at 200 rev./min on an orbital shaker. The cells were then harvested by centrifugation at 2500 *g* for 10 min. The yield of cell paste was 32 g.

All subsequent steps in the extraction were carried out at 4 °C. The cells were resuspended in 500 ml of 100 mM potassium phosphate, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 1 mM phenylmethanesulphonyl fluoride and 100 μM $ZnSO_4$, pH 7 (buffer A) and lysed by sonication; a total of 10 mg of chicken egg-white lysozyme (Sigma) was added during sonication. The insoluble cellular debris was pelleted by centrifugation at 10000 *g* for 20 min. The soluble material was applied to a 10 cm × 20 cm^2 Q-Sepharose Fast Flow (Pharmacia) column and eluted with buffer A. The flow-through (767 ml) was collected, and the fraction precipitating between 37 and 57 % $(NH_4)_2SO_4$ saturation was collected by centrifugation at 10000 *g* for 25 min. The precipitated protein was resuspended in 25 mM potassium phosphate, 1 mM DTT, 100 μM $ZnSO_4$, pH 6.6 (buffer B), to give a final volume of 22.5 ml. The preparation was then applied to a 100 cm × 2.35 cm^2 Sephacryl S-300HR (Pharmacia) column and eluted with buffer B; fractions (5ml each) were collected. The fractions were assayed for DHQ synthase activity and absorbance at 280 nm. Two resolved peaks of DHQ synthase activity were observed, the first of which had a far higher A_{280} and the second of which contained most of the activity. The fractions from the second peak containing in excess of 10 units/ml of DHQ synthase activity were pooled and applied to a 18 cm × 2.35 cm^2 hydroxyapatite (Bio-Rad HTP) column. The column was washed with 3 column vol. of buffer B, and the DHQ synthase activity was eluted by increasing the potassium phosphate concentration in a linear gradient from 25 mM to 400 mM in buffer B; fractions (12.3 ml each) were collected. The three fractions containing more than 20 units/ml of DHQ synthase activity were pooled. During the purifications, protein concentrations were measured by the method of Lowry et al. [33]; in pure preparations the method of Gill and von Hippel [34] was used. An ϵ_{280} (1mg/ml) of 0.834 was calculated on the basis of the known tryptophan, tyrosine and cysteine content of the protein [35,36].

Assay of DHQ synthase activity

Assays were routinely carried out by coupling the conversion of DAHP into DHQ to the DHQase-mediated conversion of DHQ into DHS and monitoring the increase in absorbance at 234 nm (effective ϵ_{234} 9.8×10^3 $M^{-1} \cdot cm^{-1}$ [22]). Assays were carried out at 25 °C in a volume of 1 ml and contained 12.5 Bistris propane/acetate buffer, pH 7.0, 36 μM DAHP, 125 μM NAD^+, 40 μM $ZnSO_4$ and 2 units of *S. typhi* type I DHQase. Initial rates were calculated by a least-squares fit of the first 5–10 % of the progress curve; initial-rate measurements were used to estimate the apparent K_m for NAD^+. The apparent K_m for DAHP was determined by analysis of the entire progress curve [37]. For the assay of the metal-substituted DHQ synthases zinc was omitted and EDTA was added to 50 μM to chelate adventitious metal ions in substrate, buffer and enzyme solutions. It has been shown that the rate of loss of activity of DHQ synthase caused by such concentrations of EDTA in the presence of substrate is very slow [22]. The assessment of kinetic parameters was carried out as described in the legend to Table 2 (below).

Inactivation of DHQ synthase by diethyl pyrocarbonate (DEPC)

The histidine-directed modifying reagent DEPC was diluted in 25mM Bistris propane/acetate buffer, pH 7.0, or water, the use of ethanol for the dilution of DEPC was avoided, as even a 1 % (v/v) ethanol solution resulted in significant losses of DHQ synthase activity. Modification reactions were carried out in a volume of 1 ml at 0 °C and contained 1 μM DHQ synthase domain, 25 mM bistris propane/acetate (pH 7.0) and either 0, 20, 50, 100, 150 or 200 μM of DEPC. At various time points, 50 μl aliquots were removed and quenched with 50 μl of ice-cold 100 mM imidazole (pH 7.0). The residual activity was determined by diluting 20 μl of the quenched modification mix in standard assay mix. The observed rate constants for the pseudo-first-order inactivation of DHQ synthase were calculated by estimating the gradient of the plot of log(residual activity) against $(1 - e^{-k't})/k'$, the time (t) elapsed since addition of DEPC adjusted (see [28]) for the first-order decomposition of DEPC (k') calculated as

6.52×10^{-3} min^{-1}. The second-order rate constant for the inactivation of DHQ synthase by DEPC was calculated by plotting $k_{obs.}$ against [DEPC].

The question of whether the inactivation of DHQ synthase was active-site-directed was assessed by comparison of the rate of inactivation of DHQ synthase in the presence of 32 μM DEPC in the absence and presence of substrate (DAHP, 72 μM), products (phosphate, 20 mM; DHQ, 1 mM) or cofactors (NAD^+, 250 μM; $ZnSO_4$, 200 μM) of the reaction. The pH-dependence of the reaction was investigated at five pH values over the range 6.18–7.37 with 32 μM DEPC. The stoichiometry of the inactivation was investigated by following the change in absorbance at 240 nm (ϵ_{240} of *N*-carbethoxyhistidine: 3200 $M^{-1} \cdot cm^{-1}$). The kinetic method of Tsou [38] was used to estimate the number of essential histidine residues by removing aliquots of the modification mixture at different points in the modification reaction. We attempted to show that the inactivation of DHQ synthase was due to the N-carbethoxylation of histidine residues by reversing the inactivation reaction with hydroxylamine. DHQ synthase was modified with DEPC and the reaction quenched with imidazole. The reagent and quencher were then removed by dialysis against 25 mM bistris propane/acetate buffer (pH 7). The protein was then treated in the above buffer with 0.5 M hydroxylamine/NaOH (pH 7) for 6 h at 25 °C. The hydroxylamine was then removed by dialysis against 25 mM bistris propane/acetate buffer (pH 7). Under these conditions DHQ synthase was completely inactivated, and some specific cleavage of the protein occurred. Hence the extent of reversal was monitored by determining the excess absorbance at 240 nm of the DEPC-modified and DEPC-modified-and-hydroxylamine-treated DHQ synthase solutions over an equal concentration of untreated DHQ synthase. For spectroscopy, the protein samples had been previously equilibrated in buffer containing 4 M guanidinium chloride (GdnCl) for 24 h to reduce light-scattering.

Preparation of materials for analysis of the metallochemistry of DHQ synthase

For the following experiments, adventitious bivalent cations were removed from the water, bistris propane/acetate, DAHP, NAD^+ and DHQase solutions by passing them through a column of Chelex resin (Sigma) and then storing them in polythene containers which had been washed with EDTA-containing solutions followed by metal-free water.

Determination of the rate of inactivation of DHQ synthase in the presence of the chelating agent EDTA

DHQ synthase preparations in metal-free buffers were prepared by exhaustively dialysing the protein against at least 100 vol. of 50 mM Tris/HCl buffer, pH 7.5, containing Chelex resin (Sigma), typically with three changes and for 48 h each change. The DHQ synthase preparations were then stored in sterile polycarbonate containers. The rate of inactivation of DHQ synthase in the presence of the chelating agent EDTA was determined by mixing the protein with different concentrations of EDTA in 25 mM bistris propane/acetate buffer (pH 7.0) and removing aliquots for assay at various times. The pseudo-first-order rate constant for the inactivation was determined by plotting the logarithm of the percentage remaining activity against time.

Determination of the rate constant for the dissociation of Zn^{2+} from the active site of DHQ synthase

DHQ synthase which had been stored in the presence of Chelex resin was quantitatively reconstituted with an equal concentration of $ZnSO_4$. The Zn-DHQ synthase was diluted to a final concentration of 3 μM with 25 mM bistris propane/acetate buffer, pH7.0, containing 0.5 mM $CdCl_2$. Aliquots (10 μl) were removed at various time intervals and assayed for remaining DHQ synthase activity in assay mixes containing standard concentrations of DAHP, NAD^+, *S. typhi* DHQase and 50 μM EDTA. The rate constant for the inactivation, which represents the k_{off} for the dissociation of Zn^{2+} from DHQ synthase was estimated by fitting the results by non-linear regression to an equation containing an exponential term and a constant.

Production of apo-(DHQ synthase) and its reconstitution with a range of bivalent metal ions

DHQ synthase was inactivated by mixing 400 μl of a 23 μM solution of DHQ synthase with 100 μl of a 5 mM EDTA solution. After a 1 h incubation on ice, the mixture was dialysed at 4 °C against three changes of 12.5 mM bistris propane/acetate, pH 7, 50 μM EDTA. DHQ synthase was reconstituted by mixing 20 μl of EDTA-treated enzyme, 970 μl of 12.5 mM bistris propane/acetate, pH 7, and 13 μl of 3 mM bivalent metal ion. The concentration of DHQ synthase was thus 370 nM, that of EDTA 1 μM and that of the metal ion 39 μM in the final stock solution. Assays were then performed in otherwise standard mixtures in the presence of 50 μM EDTA.

Estimation of the native M_r of the monofunctional DHQ synthase by size-exclusion chromatography

The M_r of the enzyme was estimated by size-exclusion chromatography (in 50 mM Tris/HCl, pH 7.5, containing 200mM NaCl) on a Pharmacia Superose-6 f.p.l.c. column. The column was calibrated with catalase (M_r 232000), haemoglobin (M_r 68000), BSA (M_r 67000), ovalbumin (M_r 45000), myoglobin (M_r 17000) and lysozyme (M_r 14000).

C.d. spectra of the DHQ synthase domain

C.d spectra were recorded at 20 °C in a Jobin–Yvon model CD6 spectrometer. Molar-ellipticity values were calculated using a value of 109.2 for the mean residue weight derived from the amino acid sequence of the protein [35]. Protein concentrations were 1.2–1.3 mg/ml (1 cm path length) for near-u.v. c.d spectra, and 0.5–0.65 mg/ml (0.01 cm path length) for far-u.v. c.d spectra. For the determination of the GdnCl unfolding transition, GdnCl concentrations were determined by refractive-index measurements, and the protein samples equilibrated in various GdnHCl concentrations for 24 h at 20 °C.

RESULTS AND DISCUSSION

Purification of the monofunctional DHQ synthase domain and its storage conditions

The procedure described results in a 6-fold purification of the DHQ synthase activity in approx. 50% yield (Table 1). The specific activity of the purified material was 15 units/mg, and SDS/PAGE showed that it contained a single polypeptide species of M_r 43000 (see Figure 1). A key feature of the purification is the use of Q-Sepharose as a negative column to remove unwanted proteins.

Preliminary experiments in which the monofunctional DHQ synthase activity was purified using conditions under which it bound to an initial anion-exchange column (DEAE-Sephacel) and was subsequently eluted using a salt gradient resulted in a homogeneous preparation of markedly lower specific activity,

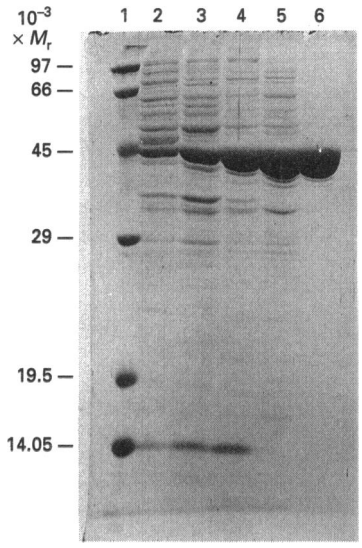

Figure 1 Purification of the *A. nidulans* AROM protein DHQ synthase domain

The SDS/polyacrylamide gel shows the purification of the monofunctional DHQ synthase domain from the overproducing strain of *E. coli*. The separating gel was 10% and the stacking gel was 5% polyacrylamide. The lanes in each case contain 15 μg of protein from: lane 2, the crude extract; lane 3, the flow-through from the Q-Sepharose column; lane 4, the fraction precipitating between 37 and 57% saturation $(NH_4)_2SO_4$; lane 5, the pooled fractions from the size-exclusion chromatography on Sephacryl S-300HR; lane 6, the pooled fractions from hydroxyapatite chromatography. M_r markers (lane 1) used were phosphorylase (97000), BSA (66000), ovalbumin (45000), carbonic anhydrase (29000), β-lactoglobin (19000) and lysozyme (14000). The gel was stained with Coomassie Blue.

even though 100 μM $ZnSO_4$ was included in the buffer. This was surprising, as it had previously been reported that the DHQ synthase activity of the pentafunctional *Neuropora crassa* AROM protein could be recovered in quantitative yield after anion-exchange chromatography in the presence of zinc ions, whereas heavy activity losses occurred in the absence of zinc ions [21]. This difference may reflect the higher pH (8.5) required for the monofunctional DHQ synthase domain to bind to anion-exchange resins. To some extent the specific activity of the anion-exchange-purified preparations could be increased after treatment with chelating agents and dialysis against solutions containing 100 μM Zn^{2+} or Co^{2+} ions. This suggests that the inactivation by anion-exchange chromatography does involve the poisoning of the active site of the enzyme by adventitious metal ions.

Steady-state kinetic parameters of the monofunctional *A. nidulans* DHQ synthase

Table 2 shows the values of the kinetic constants $k_{cat.}$, K_m (NAD^+) and K_m (DAHP) determined for the monofunctional DHQ synthase domain in this work and the corresponding values for the *E. coli* and *N. crassa* enzymes (the latter as a part of the pentafunctional AROM protein). These results indicate that the monofunctional DHQ synthase domain of the *A. nidulans* AROM protein is an efficient catalyst with $k_{cat.}$ 8 s^{-1}, K_m (DAHP) 2.2 μM and $k_{cat.}/K_m$ (DAHP) of 3.6×10^6 $M^{-1} \cdot min^{-1}$ at 25 °C and pH 7.0. The K_m (NAD^+) of the *A. nidulans* monofunctional DHQ synthase was estimated to be 3 μM under standard assay conditions. Examination of Table 2 reveals that the corresponding value for the DHQ synthase activity of the intact *N. crassa* AROM protein was estimated to be less than 200 nM. Compared with the intact AROM protein of *N. crassa*, the monofunctional DHQ synthase domain thus has a slightly lower catalytic-centre activity, a slightly higher K_m for DAHP, and a dramatically increased K_m for NAD^+ (> 15-fold).

The pH-dependence of $V_{max.}$ and $V_{max.}/K_m$(DAHP) (see Figure 2) suggests the presence of a catalytically important ionization in the enzyme mechanism. The pK_a is estimated to be 5.7 for the enzyme–substrate complex and 6.7 for the free enzyme. A caveat relating to the latter pK_a is that the apparent K_m for DAHP increases markedly with decreasing pH. Thus there is a substantial rate component in the K_m term that does not truly represent K_s. This will decrease the apparent pK_a observed, suggesting that it may in fact be higher. The basic arm of the pH profiles also show a reduction in apparent maximal velocity, but further investigation showed that this was due to a time-dependent inactivation of DHQ synthase under alkanine conditions. A similarily ionizable group with a $V_{max.}$ pK_a of 7.0 has been identified in the mechanism of the *E. coli* DHQ synthase [22].

Inactivation of DHQ synthase by the histidine-directed modifying reagent DEPC

The finding that an ionizable group with a pK_a of 6.7 was involved in the mechanism of DHQ synthase is suggestive of an active-site histidine residue. The kinetics of inactivation by the histidine-directed modifying reagent DEPC were investigated. The monofunctional DHQ synthase was found to be extremely sensitive to inactivation by DEPC (see Figure 3a), and the second-order rate constant for the inactivation was estimated to be 535 $M^{-1} \cdot min^{-1}$ at pH 7.0 and 0 °C (see Figure 3b). The pH-dependence of the inactivation was investigated by the method of Deka et al. [39] and the pK_a of the putative histidine whose modification was responsible for the inactivation was estimated to be 7.5 (results not shown). Given the contribution of rate

Table 1 Purification of the DHQ synthase domain of the *A. nidulans* AROM protein

A. nidulans DHQ synthase domain was purified from the overproducing strain of *E. coli*. The initial wet weight of the cells was 32 g. One unit of enzyme activity corresponds to 1 μmol of product formed/min at 25 °C.

Stage	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	510	1930	5000	2.59	100
Q-Sepharose	767	1200	5320	4.44	106
$(NH_4)_2SO_4$ (37–57%)	22.5	475	3200	6.73	64
S-300HR	50	150	2200	14.7	44
Hydroxyapatite	37	167	2490	14.9	50

Table 2 Kinetic parameters of the *A. nidulans* DHQ synthase domain in comparison with the corresponding values for other DHQ synthases

For the determination of the K_m for NAD^+ concentrations of NAD^+ were varied between 0.5 μM and 40 μM in otherwise standard assay mixes (fixed concentration of DAHP, 36 μM). The K_m for DAHP was determined from progress-curve analysis at described in the Experimental section and reference [37] (fixed concentration of NAD^+, 40 μM). The concentrations of DAHP ranged from 0.7 μM to to 35 μM. The K_m for DAHP and NAD^+ and $V_{max.}$ were estimated from least-squares fitting to *s/v*-versus-*s* (Hanes) plots. The S.D. of the values calculated is $\pm 5\%$. The $k_{cat.}$ value is derived from the $V_{max.}$ recorded under standard assay conditions ([NAD^+] = 13 × apparent K_m for NAD^+, [DAHP] = 16 × apparent K_m for DAHP).The values for the *N. crassa* AROM protein DHQ synthase activity were determined under the same conditions (12.5 mM Bistris propane/acetate buffer, pH 7.0, 25 °C) as the values in this work [21]; the $k_{cat.}$ was also calculated in the same manner. Those for the *E. coli* enzyme were determined in 50 mM Mops/NaOH buffer, pH 7.75, at 20 °C. Note that a K_d for the dissociation of an *E. coli* DHQ synthase–NAD^+ complex is given, rather than an apparent Michaelis constant.

Parameter	*A. nidulans* DHQ synthase (monofunctional)	*N. crassa* DHQ synthase (AROM protein)	*E. coli* DHQ synthase (monofunctional)
K_m (DAHP)	2.2 μM	1.4 μM	2.4 μM (at pH 7.75)
K_m (NAD^+)	3 μM	< 200 nM	70 nM (K_d)
$k_{cat.}$	8 s^{-1}	18 s^{-1}	60 s^{-1} (at pH 7.75)
$k_{cat.}/K_m$ (DAHP)	3.6×10^6 $M^{-1} \cdot s^{-1}$	1.4×10^7 $M^{-1} \cdot s^{-1}$	2.5×10^7 $M^{-1} \cdot s^{-1}$

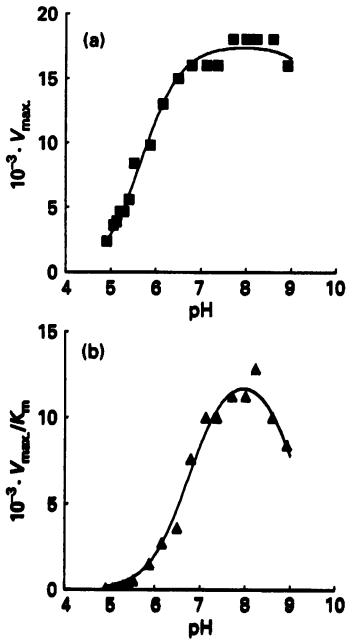

Figure 2 Dependence of DHQ synthase domain kinetic parameters on pH

The dependence of the values of the parameters $V_{max.}$ and $V_{max.}/K_m$ (DAHP) on pH were determined in bistris propane/acetate buffer (*I* 0.0125) between pH 4.91 and 9.47. The pK_a for the ionization was estimated by non-linear regression to a two ionization (bell-shaped curve) model. (**a**) Shows the pH profile for the parameter $V_{max.}$ from which the pK_a of the ionization of the enzyme–substrate complex was estimated to be 5.7. (**b**) Shows the pH profile for $V_{max.}/K_m$ from which the pK_a of the ionization of the free enzyme was estimated to be 6.75. Assays contained 20 pmol of DHQ synthase domain. Units of $V_{max.}$ are μmol of product formed·min^{-1}.

terms to the apparent K_m for DAHP, it is possible that the ionization of this putative histidine is a contributing component in the pH profile observed for $V_{max.}/K_m$ (see Figure 2b). It was also found that DAHP and, to a lesser extent, phosphate could substantially protect the enzyme against the DEPC-mediated inactivation, indicating that the inactivation was active-site-directed (see Table 3). NAD^+ was unable to protect the enzyme against inactivation (a result that mirrored the report that DAHP and not NAD^+ protected the DHQ synthase activity of the intact *N. crassa* AROM protein against EDTA-induced inactivation) and interestingly neither was DHQ. Possible explanations of these data are that either the histidine involved is protected by the phosphate moiety of DAHP and may thus be involved in its recognition, or that the histidine hydrogen-bonds to a moiety present in DAHP, but not in DHQ, such as the pyranose-ring oxygen. The stoichiometry of inactivation was also investigated using the kinetic method of Tsou [38] (see Figure 4); three of the monofunctional DHQ synthase's seven histidine residues appeared to be sensitive to modification, although the modification of one of these appeared responsible for the inactivation. Examination of an optimized alignment of the DHQ synthase sequences of *E. coli*, *Mycobacterium tuberculosis*, *A. nidulans* and *Saccharomyces cerevisiae* [40] revealed that there are three histidine residues conserved between the four sequences. It was observed that all three of these histidine residues were located within 17 residues of the 380-residue polypeptide, thus making identification of the particular histidine protected by substrate by differential peptide mapping difficult, and it was decided not to investigate this further. Treatment of the modified DHQ synthase with hydroxylamine did not result in the re-acquistion of activity (and in fact resulted in the complete inactivation of unmodified DHQ synthase). However, a substantial reduction in the absorbance at 240 nm was observed. This suggested that the increase in absorbance at this wavelength, and presumably the loss of activity when DHQ synthase was treated with DEPC, was due to the N-carbethoxylation of histidine residues.

Metal-dependence of the *A. nidulans* DHQ synthase

It had previously been shown that the purified *N. crassa* AROM protein contained one zinc atom per subunit and that preparations with reduced zinc content had a proportionately reduced DHQ synthase activity [21]. Zinc, alone of the metals tested, was capable of of fully restoring DHQ synthase activity at subnanomolar concentrations, after its inactivation by EDTA. Table 4 shows the kinetic parameters of the zinc-, cobalt-, nickel- and iron-reconstituted monofunctional DHQ synthases. Although under standard assay conditions the rate of DHQ production is greater with the zinc-reconstituted DHQ synthase than the cobalt-reconstituted enzyme, the maximal velocity observed for the cobalt-reconstituted enzyme is 25–30% higher than that observed for the former. This result parallels results obtained in previous work on *E. coli* DHQ synthase [22]. Table 4 also shows that the range of Michaelis constants for DAHP of the four enzymes varies over a smaller range than those for NAD^+. It is

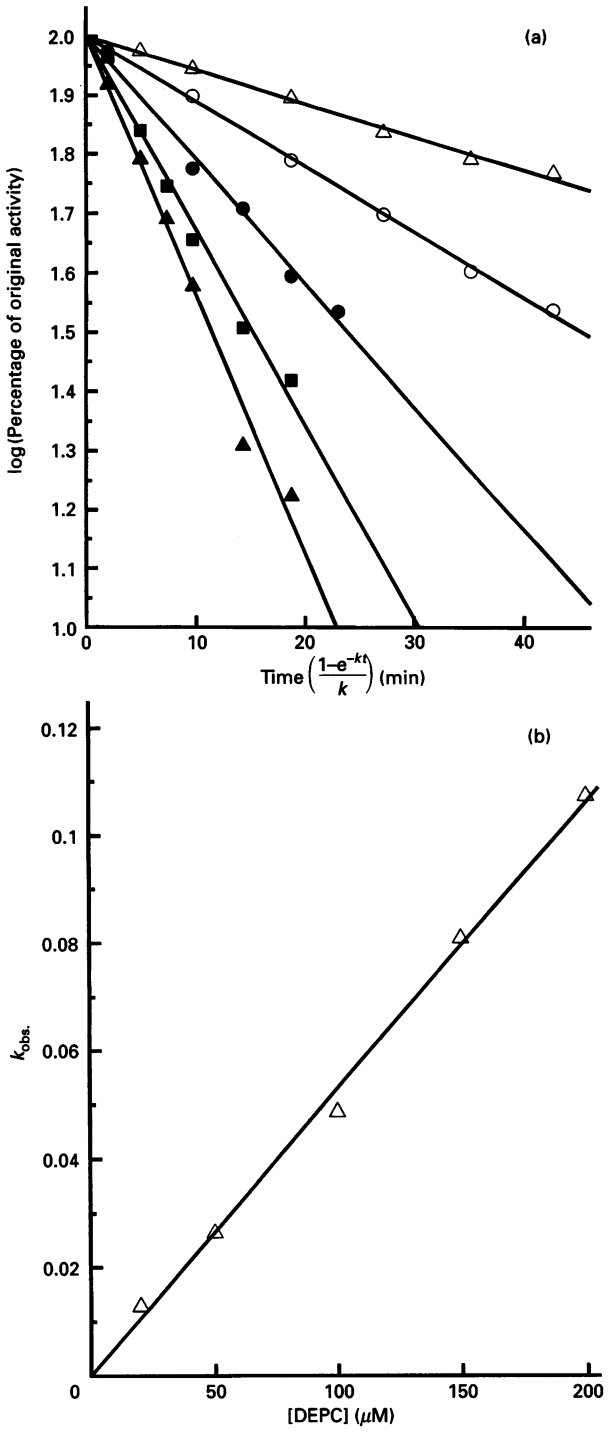

Figure 3 (a) Inactivation of DHQ synthase domain by DEPC and (b) calculation of the second-order rate constant for the DEPC-mediated inactivation of the DHQ synthase domain

(**a**) Shows pseudo-first-order plots monitoring the loss in DHQ synthase activity over corrected time when the enzyme was treated with different concentrations of DEPC. △, 20 μM; ○, 50 μM; ●, 100 μM; ■, 150 μM; ▲, 200 μM. (**b**) Shows the pseudo-first-order rate constants from (**a**) plotted against the concentration of DEPC. This experiment was carried out as described in the Experimental section. No loss in DHQ synthase activity was observed in the absence of DEPC.

noteworthy that the near 7-fold increased K_m (NAD^+) of the cobalt-reconstituted enzyme with respect to the zinc-reconstituted enzyme. The rate of NAD^+ dissociation from a zinc-reconstituted

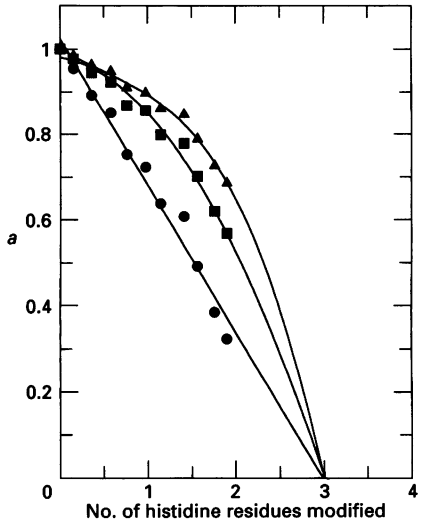

Figure 4 Estimation of the number of catalytically important histidine residues by the method of Tsou [38]

The extent of modification of DHQ synthase by DEPC was monitored by the increase in absorbance at 240 nm. The number of histidine residues modified was measured by using an absorption coefficient of 3200 M^{-1}. These results, combined with the remaining activity, were plotted as described by Tsou [38] using the relationship:

$a^{1/i} = (p-m)/p$

where a is the remaining activity when m groups have reacted, i is the number of residues(s) critical for activity, and p is the total number of groups modified at the end point of the reaction. This was estimated to be three from plotting the change in absorbance at 240 nm due to the N-carbethoxylation of DHQ synthase, and the extent of inactivation against time (results not shown). ●, $i = 1$; ■, $i = 2$: ▲, $i = 3$.

Table 3 Second-order rate constants for the inactivation of DHQ synthase by DEPC in the absence and the presence of the stated concentration of substrate, products and cofactors

Inactivation conditions	Second-order rate constant k ($M^{-1} \cdot min^{-1}$)
Standard	440
NAD^+ (250 μM)	440
DHQ (1 mM)	480
DAHP (60 μM)	145
PO_4^{3-} (20 mM)	241

E. coli DHQ synthase was reported to be 10-fold slower than that of a cobalt-reconstituted enzyme [22].

Inactivation of DHQ synthase by EDTA and metal ions

In the presence of EDTA at 0 °C, the isolated DHQ synthase domain was found to be rapidly inactivated compared with the DHQ synthase activity of the *N. crassa* AROM protein. In contrast with the *N. crassa* AROM protein [21] and *E. coli* DHQ synthases [22], where the inactivation by EDTA follows second-order kinetics, the rate of inactivation of the *A. nidulans* isolated DHQ synthase domain was not directly proportional to the EDTA concentration. Extrapolation of a least-squares fit of the rates of inactivation in the presence of EDTA to zero EDTA concentration suggested a basal rate of inactivation of approx.

Table 4 Kinetic properties of metal-ion-substituted DHQ synthases

The apparent $k_{cat.}$ and K_m values of the metal-substituted DHQ synthase for DAHP and NAD^+ were determined as described in the legend to Table 2, except that rates were recorded for concentrations of NAD^+ up to 80 μM for zinc- and copper-substituted DHQ synthases and 200μM for the iron- and nickel-substituted DHQ synthases. The ratio of the catalytic activity of the metal-substituted DHQ synthases of the *A. nidulans* monofunctional DHQ synthase domain is compared with those previously reported for the *E. coli* enzyme [22]. M^{2+} represents the bivalent metal ion, either Zn^{2+}, Co^{2+}, Fe^{2+} or Ni^{2+}.

Metal ion	K_m (NAD^+)	K_m (DAHP)	$k_{cat.}$	$\frac{k_{cat.}(M^{2+})}{k_{cat.}(Zn^{2+})}$ (*E. coli* DHQ domain)	$\frac{k_{cat.}(M^{2+})}{k_{cat.}(Zn^{2+})}$ (*A. nidulans* synthase
Zn^{2+}	3.4 μM	1.7 μM	8 s^{-1}	1.00	1.00
Co^{2+}	23 μM	2.4 μM	10 s^{-1}	1.25	1.89
Fe^{2+}	3.5 μM	4.5 μM	1.4 s^{-1}	0.18	N.R.
Ni^{2+}	16 μM	2.0 μM	.3 s^{-1}	0.16	0.43

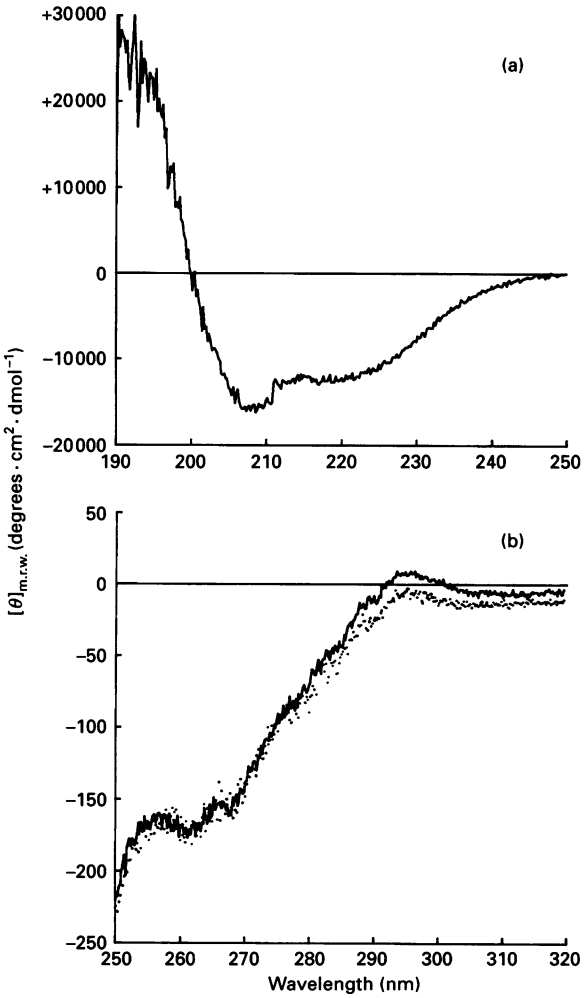

Figure 5 Far- (a) and near- (b) u.v. spectra of the DHQ synthase domain

——, Zn^{2+}-DHQ synthase; – – – –, Co^{2+}-DHQ synthase). Spectra were recorded in 10 mM potassium phosphate, 90 mM KCl, pH 7.0, at 20 °C.

0.045 min^{-1} (results not shown). In the absence of substrate an excess of cadmium ions led to the rapid inactivation of the DHQ synthase. Using a model permitting free exchange of the zinc and cadmium it was estimated that the k_{off} for the dissociation of Zn from DHQ synthase was 0.036 min^{-1}. The basal rate of inactivation observed in the experiments where DHQ synthase was inactivated with EDTA could thus be attributed to the first order dissociation of the metal from the active site.

Determination of the quaternary structure of the monofunctional DHQ synthase

Size-exclusion chromatography using a column calibrated with globular proteins suggests a native M_r of 43000. As the M_r of the DHQ synthase domain inferred from the nucleotide sequence is also 43000, this indicates that the isolated domain is monomeric with a globular well-ordered structure.

Solution conformation of the isolated DHQ synthase domain

Figures 5(a) and 5(b) respectively show that the far- and near-u.v. spectra of the DHQ synthase domain. The results suggest that the secondary-structure content of the domain is about 75%, with approx. 50% of the residues being in an α-helical and 25% in a β-sheet conformation [41]. The near-u.v. spectra of the DHQ synthase domains in zinc- and cobalt-containing buffers was found to be essentially superimposable. The ellipticity at 220 nm of DHQ synthase domain was determined in GdnCl concentrations from 0 to 6 M. The midpoint in the apparently single transition representing the loss of secondary structure is at 1 M GdnCl.

Conclusions

The results reported here show that the DHQ synthase domain of the *A. nidulans* AROM protein remains an efficient catalyst when it is expressed as a monofunctional domain. It appears that in this state it is monomeric, as is the individual DHQ synthase of *E. coli* [18]. The intact pentafunctional AROM protein of *N. crassa* has been demonstrated to be dimeric [42,43], a result predicted by earlier genetic work [44] which also suggested that the regions of the AROM protein involved in dimerization were the shikimate dehydrogenase and DHQ synthase domains [45]. Given that the catalytic efficiency of the monofunctional DHQ synthase domain appears only slightly reduced from that of the entire protein and that this domain has been implicated in dimerization, it might have been expected that the isolated DHQ synthase domain would be dimeric. It has recently been reported that the DHQase domain of the AROM protein is monomeric when individually overproduced, and its $k_{cat.}/K_m$ value is much

reduced from that of the corresponding activity in the intact AROM protein of the related species *N. crassa* [12,21]. Interestingly the *E. coli* [46] and *S. typhi* DHQases [28] are dimeric. These results fit into a picture which has been sketched by reports on the enzymology of the CAD multifunctional protein in pyrimidine synthesis. In this system all of the constituent domains are independently functional, but, like the AROM protein, the efficiency of some of the activities is markedly reduced, whereas others are far less affected when engineered to be overproduced individually [47].

There do seem to be significant changes in some of the properties of the separately expressed *A. nidulans* monofunctional DHQ synthase and those of the corresponding activity in the intact *N. crassa* AROM protein. Firstly the NAD^+ prosthetic group is not bound as tightly in the isolated domain as in the native pentafunctional protein as judged by the increased K_m value. Similarly the active-site metal is less tightly bound, since the half-life of activity in the presence of EDTA is much reduced in the monofunctional DHQ synthase; EDTA inactivation does not follow second-order kinetics suggesting that the metal readily dissociates from the active site.

The DEPC-inactivation experiments are the first chemical-modification data reported for any DHQ synthase and suggest that an active-site histidine may be present. This may be the ionizable group in the mechanism indicated by the pH–activity studies. There are three conserved histidine residues in the four DHQ synthase sequences so far reported, all within the same region of the polypeptide chain. The substrate DAHP and, to a lesser extent, phosphate, protect against both the DEPC-mediated inactivation of the monofunctional *A. nidulans* DHQ synthase, and the EDTA-mediated inactivation of the DHQ synthase activity of the native AROM protein of *N. crassa* [21 , whilst NAD^+ protects against neither inactivation.

The overall picture is that the monofunctional DHQ synthase domain has lost some of the affinity for its ligands compared with the pentafunctional protein. Currently we are unable to make the ideal comparison with the *A. nidulans* AROM protein, as this has only been overproduced and purified in a mutant form lacking the DHQ synthase activity [48,13] . We are currently attempting the overproduction of the entire *A. nidulans* AROM protein in *E. coli* to facilitate such a comparison.

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