Conformational changes and the role of metals in the mechanism of type II dehydroquinase from *Aspergillus nidulans*

Joanna R. BOTTOMLEY*, Alastair R. HAWKINS† and Colin KLEANTHOUS*‡

*School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K., and †Department of Biochemistry and Genetics, Catherine Cookson Building, University of Newcastle, Newcastle upon Tyne NE2 4HH, U.K.

We have investigated the involvement of metal ions and conformational changes in the elimination reaction catalysed by type II dehydroquinase from *Aspergillus nidulans*. Mechanistic comparisons between dehydroquinases and aldolases raised the possibility that, by analogy with type II aldolases, type II dehydroquinases may require bivalent metal ions for activity. This hypothesis was tested by a combination of metal analysis, effects of metal chelators and denaturation/renaturation experiments, all of which failed to show any evidence that type II dehydroquinases are metal-dependent dehydratases. Analysis of native and refolded enzyme by electron microscopy showed that the dodecameric type II enzyme from *A. nidulans* adopts a ring-like structure similar to that of glutamine synthase, suggesting an arrangement of two hexameric rings stacked on top of one another. Evidence for a ligand-induced conformational change came from both chemical modification and proteolysis experi-

ments. Inactivation data with the arginine-specific reagent phenylglyoxal indicated that, at pH 7.5, two arginine residues are modified: one modification displays affinity-labelling kinetics and has a 1:1 stoichiometry, while the other displays simple bimolecular kinetics and a stoichiometry of 2:1. The labelling at the affinity site is markedly enhanced by the addition of ligand, implying that this active-site residue is further exposed to modification by phenylglyoxal as a result of a ligand-induced conformational change. A combination of proteolysis and electrospray MS experiments identified the site of affinity labelling as Arg-19. The highly conserved N-terminal region encompassing Arg-19 of type II dehydroquinase was found to be particularly susceptible to proteolytic cleavage. Limited digestion with proteinase K inactivates the enzyme, although the type II oligomeric structure is retained, and ligand binding partially protects against this inactivation.

INTRODUCTION

The enzyme 3-dehydroquinase (DHQase; EC 4.2.1.10) catalyses the dehydration of 3-dehydroquininate to 3-dehydroshikimate (Scheme 1) and is a central step in two distinct metabolic pathways: the biosynthetic shikimate pathway of bacteria, fungi and plants [1–3] and the catabolic quinate pathway, thus far characterized in the fungi *Neurospora crassa* and *Aspergillus nidulans* as well as the bacterium *Amycolatopsis methanolica* [4–6]. In a biosynthetic context, 3-dehydroshikimate is converted into the central metabolite chorismate, which is the precursor for a wide range of aromatic compounds such as aromatic amino acids, ubiquinone and vitamin E. In a catabolic context, 3-dehydroshikimate is further dehydrated to protocatechuate,

which is metabolized through the β -ketoadipate pathway to yield acetyl-CoA. Since the shikimate pathway is absent in mammals, enzymes in this pathway are potential targets for antimicrobial agents and herbicides [7].

DHQases have been classified into type I and type II enzymes based on a number of properties, such as amino acid sequence, subunit molecular mass, oligomeric structure and thermal stability [8]. Type I enzymes [9–11] and type II enzymes [6,12–15] have been identified in both shikimate and quinate pathways from different micro-organisms. *Escherichia coli* DHQase is typical of a type I enzyme. It is dimeric, has a subunit molecular mass of 27.4 kDa [9,16], is heat labile and catalyses the elimination of 3-dehydroquininate to 3-dehydroshikimate with *syn* stereochemistry [17]. Two active-site groups have been identified in the mechanism of the *E. coli* type I DHQase: Lys-170 forms a Schiff base with the substrate keto group and plays a purely catalytic role as an electron sink during the elimination reaction [16,18,19], and His-143 has been implicated as a general acid/general base involved in both Schiff base formation and proton abstraction [19,20]. The importance of a number of other amino acid residues originally identified by chemical modification experiments [21,22] has subsequently been discounted by mutagenesis studies [19]. In addition, tryptophan fluorescence equilibrium binding measurements have suggested that a conformational change occurs in the mechanism of the type I DHQases [19].

Type II DHQases have a much smaller subunit molecular mass than type I enzymes (16–18 kDa) and they oligomerize into

Scheme 1 Dehydration reaction catalysed by the two classes of DHQase

The diagram highlights the changes in ring conformation between substrate and product, as well as the different stereochemical courses catalysed by the two classes of enzyme.

Abbreviations used: DHQase, 3-dehydroquinase; ESI-MS, electrospray ionization MS; Gn.HCl, guanidine hydrochloride; ICP-AES, inductively coupled plasma atomic emission spectrometry; NTA, nitrotriacetic acid; PGA, phenylglyoxal; PVDF, poly(vinylidene difluoride).

‡ To whom correspondence should be addressed.

a dodecameric structure that is very heat stable [4,8]. They catalyse the elimination of water from 3-dehydroquinone with *trans* stereochemistry [23,24] and without the involvement of covalent intermediates [8]. Few mechanistic details are known about this class of enzyme. Recent chemical modification experiments on the *Streptomyces coelicolor* type II DHQase have highlighted Arg-23 as being particularly reactive towards phenylglyoxal (PGA) modification [25], although it remains unclear whether or not this residue is within the active site and what role it may play in the mechanism.

The two types of DHQase represent an unusual case of convergent evolution [26]. They share no obvious sequence identity (although a putative substrate binding motif has been described by Kleanthous et al. [21]), they have very different physical properties and they catalyse the conversion of 3-dehydroquinone to 3-dehydroshikimate by completely different mechanisms. Indeed, the only point of convergence between the type I and type II DHQase families is the fact that they catalyse the same reaction. A key step in the mechanism of the DHQases is the stabilization of a putative carbanion intermediate following abstraction of either the *pro-R* proton in the case of the type I enzymes or the *pro-S* proton in the case of the type II enzymes (Scheme 1). In the type I enzymes the resulting carbanion is stabilized through Schiff base formation. What is not clear, however, is how such a putative intermediate is stabilized in the type II class of DHQases. In trying to address this issue we looked towards other enzymes from which we could draw mechanistic comparisons. Interesting analogies can be drawn between the disparate DHQase enzymes and the fructose-1,6-bisphosphate aldolases, which catalyse the interconversion of fructose 1,6-bisphosphate with dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. As with the DHQases, a central feature of the mechanism of the aldolases is the stabilization of a carbanion intermediate, and this is accomplished by two completely different mechanistic routes; type I enzymes are found in higher eukaryotes and stabilize this intermediate through a Schiff base [27,28] analogous to the type I DHQase enzymes, whereas type II aldolases, found in bacteria and fungi, utilize a tightly bound metal ion as an electrophilic catalyst ([29] and references therein). The possibility therefore exists that type II DHQases, by analogy with type II aldolases, catalyse the dehydration of 3-dehydroquinone to 3-dehydroshikimate through bivalent metal-mediated electrophilic catalysis.

In the present work we address two issues relating to the mechanism of type II DHQases: (i) the role of metal ions, and (ii) the involvement of conformational changes, as deduced from chemical modification and limited proteolysis experiments. As part of this study we also used electron microscopy to investigate the gross quaternary structure of the dodecameric type II enzyme from *A. nidulans*.

MATERIALS AND METHODS

Chemicals

Ammonium 3-dehydroquinone was prepared by the method of Grewe and Haendler [30]. Other chemicals were of reagent grade or better and were used without further purification.

Expression and purification of *A. nidulans* type II DHQase

The purification of the enzyme followed a procedure modified from those previously described [5,31]. Type II DHQase of *A. nidulans* was constitutively overexpressed in *E. coli* AB1157 which had been transformed with the plasmid pKK9 (derived from pKK233-2; Pharmacia) containing the *qutE* gene encoding

the type II enzyme subcloned from pEH1 [32]. Overexpressed type II DHQase was purified from 5-litre cultures of *E. coli* AB1157 containing pKK9 which had been grown for 16 h with vigorous aeration in LB broth containing ampicillin (50 µg/ml) at 37 °C. The cells were harvested by centrifugation at 16300 g for 15 min and resuspended in a minimal volume of TEM buffer [Tris/HCl buffer (50 mM, pH 7.5) containing EDTA (1 mM), β-mercaptoethanol (1 mM)] and the protease inhibitor PMSF (1.2 mM) at 4 °C and broken using a French press [~ 62–100 kPa (9000–10000 psi)]. Following incubation with DNase and RNase (0.1–0.2 mg per 30 ml of extract) at 37 °C for 30 min, the cell debris was removed by centrifugation at 12000 g for 30 min at 4 °C. The supernatant was heated to 70 °C for 15 min with continuous stirring and then placed on ice for 60 min. This treatment precipitates the endogenous type I enzyme of *E. coli*. Precipitated protein was removed by centrifugation at 12000 g for 45 min at 4 °C and the supernatant was dialysed against TEM buffer containing KCl (50 mM). The extract was applied to a DE-52 anion-exchange column (50 ml) equilibrated with the same buffer at 4 °C. The column was washed extensively with the loading buffer (1 ml/min) until the absorbance at 280 nm fell below 0.1 and then eluted with a 500 ml KCl gradient (50–500 mM in TEM buffer). DHQase-containing fractions were pooled and dialysed against TEM buffer containing KCl (50 mM), concentrated by ultrafiltration using an Amicon concentrator and loaded (1 ml/min) on to a Sephacryl S-300 gel-filtration column (500 ml) equilibrated with TEM buffer containing KCl (50 mM) at 4 °C. Pooled DHQase-containing fractions were concentrated by ultrafiltration, dialysed against TEM buffer and stored at –20 °C. The typical yield of protein from this purification scheme was ~ 30 mg and the protein was judged to be pure by SDS/PAGE.

Protein, activity determinations and kinetic experiments

Protein content was determined by amino acid analysis in conjunction with the known composition of the protein [33]. Enzymic activity was monitored spectrophotometrically by measuring the formation of product, 3-dehydroshikimate, at pH 7.0 and at 234 nm essentially as described by Kleanthous et al. [8]. Kinetic experiments involving the alteration of the substrate concentration were also as previously described [8].

Metal analysis

(i) Preparation of samples

Plasticware was used throughout the preparation of samples. Before use, all vessels were washed with conc. HCl/conc. HNO₃ (3:1, v/v) and then with analytically pure water (Fluka). Dialysis tubing (10 kDa cut-off) was always freshly prepared in the presence of EDTA (1 mM), boiled and then washed extensively with metal-free water [34]. Samples of purified enzyme were exhaustively dialysed against several changes of low-concentration buffer (10–20 mM Tris/HCl, pH 7.5) to reduce sources of contamination. In some experiments either EDTA (1 mM) or KCl (100 mM) was also included. All buffers and standards were made up with analytically pure water (Fluka). The final dialysis buffers were retained and used to correct for background contamination.

(ii) Qualitative analysis by simultaneous/sequential inductively coupled plasma atomic emission spectrometry (ICP-AES)

Metal analysis was initially carried out using simultaneous/sequential ICP-AES (Thermo Jarrell Ash) [35]. Protein samples

of 10–15 ml (1–10 mg/ml) were used to scan for the presence of Mg, Al, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn and Cd.

(iii) Quantitative analysis by atomic absorption spectrometry

Protein samples (0.1–0.8 mg/ml) were dialysed exhaustively into a variety of buffers made up in metal-free water (Fluka). Buffers typically contained Tris/HCl (20 mM, pH 7.5), and for some analyses also contained EDTA (1 mM) and KCl (100 mM). Samples were analysed for zinc content using a graphite furnace atomic absorption spectrometer (Philips PU9200). Automated analysis of 1–100 μ l samples consisted of a drying step to remove water (110 °C for 125 s), an ashing step to remove organic material (400 °C for 20 s) followed by atomization (1600 °C for 2 s and 1800 °C for 2 s). Samples were measured against standards of known concentration.

Chelators

The effects of increasing concentrations (ranging from 0 to 10 mM) of nitrilotriacetic acid (NTA), EDTA and EGTA on the type II DHQase were examined using the standard assay mixture described above. In other experiments, type II DHQase was incubated with EDTA (10 mM) on ice and samples were removed at specified time intervals to monitor for the loss of activity. Enzymic activity was also monitored over an extended period of dialysis against Tris/HCl (50 mM, pH 7.0) containing a high concentration of EDTA (10 mM).

Denaturation and renaturation of type II DHQase

DHQase (0.5 mg/ml) was incubated in Tris/HCl (10 mM, pH 7.5) containing EDTA (1 mM) and guanidine hydrochloride (Gn.HCl; 7 M) for 1 h, which was sufficient to fully unfold the protein as judged by CD (see below), and then fractionated by gel-filtration chromatography using a Sephadex G-25 column (50 ml) equilibrated in the same buffer at room temperature. Protein-containing fractions were identified by absorbance at 280 nm, pooled and dialysed exhaustively against Tris/HCl (10 mM, pH 7.5) containing EDTA (1 mM) at 4 °C, to remove the denaturant. The buffer was prepared with metal-free water and all apparatus was acid washed as described above. Controls for this experiment were samples of enzyme which were passed through the Sephadex G-25 column in the same buffer but omitting the denaturant.

CD analysis

This was carried out at 20 °C using a Jasco spectropolarimeter and quartz cells of 0.1 cm pathlength. The protein concentrations were typically 0.02 mg/ml. The unfolding of the type II DHQase by Gn.HCl has been reported previously [8]. The time needed to unfold the enzyme was investigated in this study by monitoring the loss of $[\theta]_{225}$ as a function of time at a concentration of Gn.HCl that is known to fully unfold the enzyme (7 M). The secondary structure composition of the refolded enzyme was deduced as previously described [8].

Quaternary structure

(i) Molecular mass determination

Samples of native type II DHQase and type II DHQase that had been refolded following denaturation by Gn.HCl were applied to a calibrated FPLC gel-filtration column (10–300 K diol bonded silica; Waters). The column was eluted with Tris/HCl (50 mM,

pH 7.5) at 0.5 ml/min and room temperature and calibrated with the following standard proteins: ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa), type I DHQase (55 kDa) and chymotrypsin A (25 kDa).

(ii) Scanning electron microscopy

Protein samples were prepared essentially as described by Harris [36]. Type II DHQase (typically 1 mg/ml) in Tris/HCl (10 mM, pH 7.5) containing 2% ammonium molybdate and 0.1–0.2% poly(ethylene glycol) (molecular mass 1000–10000 Da) was spread over a freshly cleaved piece of mica. Excess liquid was removed and the sample allowed to air-dry prior to coating with carbon *in vacuo* (Edwards Vacuum Evaporator, model 12E6). The carbon film was then separated from the mica by submerging in negative stain (ammonium molybdate and uranyl acetate). Finally, uncoated copper grids (400 mesh) were brought beneath the floating carbon film and the sample allowed to air-dry. Specimens were viewed by scanning electron microscopy using a Jeol JEM-100CX electron microscope.

Chemical modification of arginine residues with PGA

Modification of arginine groups typically involved incubating type II DHQase (0.8 mg/ml; 48 μ M) in Hepes buffer (50 mM, pH 7.5) at 25 °C with PGA (0–10 mM) dissolved in Hepes buffer (50 mM, pH 7.5). Time-course measurements involved removing samples (10 μ l) and quenching the modification by dilution prior to assaying for residual enzymic activity using the standard assay (see above). In some experiments a substrate/product mixture (molar ratio 1:15; [19]) was included in the incubation. The effect of pH on the modification of the enzyme was analysed using Hepes buffer over the pH range 7.0–9.4.

The stoichiometry of PGA modification was analysed using [14 C]PGA (final specific radioactivity 1.85 μ Ci/ μ mol; Amersham) and the incorporation of radioactivity was monitored by trichloroacetic acid precipitation of the protein on to filter discs [37]. Enzyme (0.5 mg/ml) was incubated with [14 C]PGA (10.8 mM) in Hepes buffer (50 mM, pH 7.5) at 25 °C. Samples were removed periodically for determination of incorporated label (2 $\times$ 20 μ l) and residual enzymic activity (10 μ l). The incorporation of label was analysed by first quenching the reaction by dropping the samples on to glass filter discs (2.5 cm) and then immersing them in trichloroacetic acid (5%) at 4 °C. The discs were then washed with an equal volume of trichloroacetic acid (5%) and then with cold ethanol/diethyl ether (1:1, v/v) for 10 min. Finally, the discs were rinsed in ether and allowed to air-dry, and radioactivity was counted in scintillation fluid (4.5 ml; Pico-fluor) using an LKB Wallac 1214 Rackbeta Liquid Scintillation Counter. Background counts which resulted from residual radioactive PGA remaining on the discs after washing were subtracted from the sample.

Electrospray ionization MS (ESI-MS)

ESI-MS was used to monitor the modification of type II DHQase by PGA and to identify peptide fragments resulting from proteolytic cleavage of the enzyme. Protein samples taken at specified time points (see the Results section) from PGA modification experiments were first fractionated by gel-filtration chromatography using a Sephadex G-25 column (50 ml) equilibrated in Hepes buffer (50 mM, pH 7.5) to stop the reaction and remove excess reagent, and then dialysed into either water or a low concentration of Tris/HCl buffer (10 mM, pH 7.5) at 4 °C. Protein from proteolytic digests was analysed directly (see below). Samples from both types of experiment were analysed either on

a Finnegan MAT TSQ700 (carrier solvent methanol/water/acetic acid, 1:1:0.01, by vol.) or on a Fisons VG Platform (carrier solvent acetonitrile/water/formic acid, 1:1:0.001, by vol.) electrospray mass spectrometer at a flow rate of 5–10 $\mu\text{l}/\text{min}$. In each case, the sample was first diluted into the carrier solvent prior to analysis (~ 30 pmol/run). Horse heart myoglobin was used as a calibrant for both spectrometers. Data were acquired over the range m/z 700–1300, with five or more scans averaged and processed using the supplier's software.

Proteolytic digestions

Type II DHQase was treated with a number of proteases, including proteinase K (in Tris/HCl buffer, pH 8.5, at 37 °C), chymotrypsin (in Tris/HCl buffer, pH 8.5, at 37 °C), thermolysin (in Hepes buffer, pH 8.5, at 60 °C) and papain (in Tris/HCl buffer, pH 8.5, at 37 °C). With the exception of proteinase K, the ratio of substrate protein to protease was 25:1 (w/w) and incubations were for between 1 and 6 h. For proteinase K a much lower ratio was used (500:1, w/w) and the digestion was only for 1 h. The fragments from these digestions were resolved by Tricine SDS/PAGE [38] and blotted on to poly(vinylidene difluoride) (PVDF) membranes (Immobilon-P; Millipore) for N-terminal sequence analysis. The relative molecular masses of peptide fragments were identified by running a set of low-molecular-mass markers (MW SDS-17S; Sigma) in the gels (range 17–2.5 kDa). Bands on the blots were identified by staining with Coomassie Blue (< 1 min).

In a separate series of experiments, digestions with proteinase K were carried out at a protein/protease ratio of 500:1 (w/w) and the time course of inactivation by proteolysis was determined over 60 min, using the standard assay described above, in the absence and presence of a substrate/product equilibrium mixture (1 mM). Proteolysis was quenched by freezing the samples (in a solid CO_2 /ethanol bath), which were then analysed directly by ESI-MS as described above.

Proteinase K digestion was also used to identify the arginine residue that is modified early on in PGA modification experiments (k_{fast} ; see below). Type II DHQase (5.3 mg/ml) was inactivated with PGA (10 mM) as described above. Excess reagent was removed by gel filtration in Tris/HCl buffer (10 mM, pH 8.5), the samples were concentrated by lyophilization and the protein was then incubated with proteinase K (500:1) at 37 °C for either 30 or 50 min. Proteolysis was quenched by freezing the samples in a solid CO_2 /ethanol bath and then analysing them directly by ESI-MS.

RESULTS AND DISCUSSION

Little is known of the mechanism of type II DHQases. The work reported in the present paper sets out to address two areas that have not been investigated, the role of metal ions as electrophilic catalysts and the involvement of conformational changes. The source of the enzyme was a strain of *E. coli* (AB1157) containing a plasmid that constitutively expresses recombinant *A. nidulans* type II DHQase; the enzyme was then purified using a procedure slightly modified from those reported previously [5,31]. Kinetic parameters for the purified enzyme were similar to those reported by Kleanthous et al. [8]: k_{cat} , $1250 \pm 59 \text{ s}^{-1}$ (literature value 1300 s^{-1}); K_{m} , $147 \pm 4 \mu\text{M}$ (literature value $150 \mu\text{M}$).

A. nidulans type II DHQase and the involvement of metal ions in the mechanism

We addressed the issue of metal-mediated catalysis in the mechanism of the type II DHQase from *A. nidulans* using three

separate approaches: metal analysis, the effect of metal chelators, and gel filtration of the unfolded protein in the presence of a chelator followed by refolding of the enzyme.

(i) Metal analysis

Metal analysis was conducted in two stages: first, a multi-elemental scan using ICP-AES (see the Materials and methods section) was carried out to identify metal(s) associated with protein samples, and secondly by atomic absorption to quantify metal content. ICP-AES analysis identified Ca^{2+} , Co^{2+} , Mg^{2+} and Zn^{2+} as the most consistently occurring elements in protein samples [39]. The poor sensitivity of ICP-AES makes quantification difficult and so atomic absorption using a graphite furnace was used to estimate the stoichiometry of metal/protein. Atomic absorption showed that Ca^{2+} , Co^{2+} and Mg^{2+} were present in very low amounts and likely to be contaminants acquired either during sample preparation or during the ICP-AES analysis. Zn^{2+} , however, remained at measurable levels in many of the samples that were analysed, even when EDTA (1 mM) and KCl (100 mM) were present in the dialysis buffer. Four independently purified preparations of type II DHQase were analysed and in almost every case Zn^{2+} was measurable in the protein samples. However, the amount of Zn^{2+} present was always substoichiometric; duplicate analyses of each purification (eight samples were analysed) yielded a stoichiometry of 0.107 mol of Zn^{2+} /mol of protein subunit (S.E.M. ± 0.066). Samples of enzyme that had been spiked with a stoichiometric amount of Zn^{2+} and then dialysed showed similar amounts of metal associated with the protein as untreated samples. In conclusion, quantitative metal analysis of type II DHQase from *A. nidulans* consistently shows substoichiometric amounts of Zn^{2+} associated with the enzyme, suggesting that activity is not dependent on a tightly bound metal ion.

(ii) Effect of metal chelators on the activity of type II DHQase

Metal analysis had indicated that there is unlikely to be a metal centre in type II DHQase from *A. nidulans*. Such analyses can often be complicated, however, by the possibility that the real metal content of the protein may be masked (e.g. ashing may not release all the metal present in the protein). We therefore investigated the effect of chelators on the activity of the type II enzyme, since these are often used to define the involvement of metal centres in metalloenzymes [40]. It has previously been noted that low concentrations of EDTA do not inhibit the enzyme [8]. However, since some metalloenzymes can retain their metals in dilute solutions of chelators [41], the effect of chelators on the activity of type II DHQase was reinvestigated. Three chelators, NTA, EDTA and EGTA (in the concentration range 0–10 mM), were studied for their effect on DHQase activity in a typical type II assay (see the Materials and methods section). Neither NTA nor EGTA affected the activity of the enzyme [39]. The presence of EDTA in the assay, as previously reported, did not affect the activity of the enzyme at low concentrations (< 2 mM), but significant inhibition was observed at higher concentrations. The kinetics of inhibition by EDTA were complex, however, as Lineweaver–Burk plots suggested that the chelator was behaving as a mixed, principally non-competitive, inhibitor of the enzyme [39]. In other experiments, EDTA (10 mM) was preincubated with the enzyme for several hours or the enzyme was dialysed against the chelator over several days. In each case, full activity could be recovered simply by dilution of the enzyme into a standard assay (results not shown). The effect of metals added directly to enzyme assays was also analysed to see if activity could be elevated. The addition of Zn^{2+} (1 mM), Mg^{2+}

Scheme 2 Conserved core sequence of a nucleotide binding region at the C-termini of type II DHQases

Hydrophobic residues are indicated by stippling. The secondary structure elements corresponding to this motif are shown above the alignment. *S. coelicolor*, *Streptomyces coelicolor*; *N. crassa*, *Neurospora crassa*; *M. tuberculosis*, *Mycobacterium tuberculosis*; *A. pleuropneumoniae*, *Actinobacillus pleuropneumoniae*.

(10 mM) and Co^{3+} (10 mM) all resulted in severe inhibition of type II DHQase activity (results not shown), consistent with the idea that they do not play a role in the mechanism of the enzyme.

(iii) Renaturation of type II DHQase in the presence of EDTA

A final series of experiments aimed at investigating the possible involvement of metals in the mechanism of type II DHQase required that the enzyme be refolded in the presence of a chelator following gel filtration of protein subunits in the unfolded state. The experiment also allowed us to determine whether any other tightly bound cofactors might be needed for activity of the enzyme, since these should be removed during the course of the experiment. The suggestion that a tightly bound cofactor might be involved in the mechanism arose from alignments of all type II DHQase sequences, which showed that the C-terminus contains half the core sequence of a typical dinucleotide binding motif (Scheme 2) [42].

Kleanthous et al. [8] have previously reported that the type II enzyme shows a complex Gn.HCl denaturation profile, with two or three discernible transitions as deduced by CD, and is fully denatured in 6 M Gn.HCl . In these experiments the enzyme was incubated with the denaturant for up to 24 h prior to measurement of the near-UV CD signal, and activity could not be recovered by dilution. In the present study, preliminary experiments showed that incubation of the enzyme with 7 M Gn.HCl resulted in the complete loss of secondary structure (as deduced by CD) within 1 min and that enzyme which had been denatured for < 1 h could be refolded by dialysis to yield essentially wild-type levels of activity. Subsequent experiments capitalized on these observations.

As described in the Materials and methods section, type II DHQase was unfolded using 7 M Gn.HCl in the presence of 1 mM EDTA, fractionated by gel-filtration chromatography under the same conditions and then refolded by dialysis. Control samples were treated in the same manner but Gn.HCl was not included in any of the buffers. The complete experiment was performed on two separate preparations of enzyme and, following refolding of the enzyme by dialysis, the recovered specific activity was 79 % and 91 % respectively. CD ($\theta_{190-260}$) indicated ~ 65 % α -helix in the refolded protein, compared with 75 % reported for the native enzyme [8].

The native oligomeric structure of the type II enzyme from *A. nidulans* is a dodecamer with a weight averaged molecular mass of 190 kDa [8]. In addressing the issue of the recovery of native

Figure 1 Electron micrograph of type II DHQase from *A. nidulans*

The enzyme was prepared for electron microscopy by negative staining as described in the Materials and methods section. The magnification is $\times 120\,000$ and the bar represents 1000 Å (100 nm).

protein following denaturation, two methods were used to determine whether refolded enzyme had the same oligomeric structure as wild-type enzyme: gel-filtration chromatography and electron microscopy. Refolded enzyme was eluted from a calibrated (10–300 kDa) gel-filtration column at the same elution volume as undenatured enzyme and in the mass range 200–230 kDa, suggesting that the refolded enzyme was dodecameric (results not shown). The three-dimensional structure of any type II DHQase has yet to be reported, and so it was of interest to investigate the gross quaternary structure of native and refolded enzyme by scanning electron microscopy. Negative staining of protein samples indicated the presence of hollow ring structures (Figure 1) reminiscent of other dodecameric enzymes such as glutamine synthase [43]. The overall dimensions of these rings were ~ 250 Å (25 nm) in diameter and ~ 120 Å in height. The ring structure was not present when protein was omitted from the staining procedure. Furthermore, the same ring structures were observed in samples that had been refolded from denaturant, in agreement with the gel-filtration results, indicating the recovery of the quaternary structure of the enzyme (results not shown).

These final experiments showed that active and refolded type II DHQase could be recovered following gel filtration under denaturing conditions in the presence of a chelator, suggesting that neither a metal nor a tightly bound cofactor are involved in the mechanism. The question remains, however, as to the functional or structural role of the partial NAD^+ -binding motif in this class of enzyme.

Ligand-induced conformational changes analysed by arginine modification and proteolysis experiments

The ring conformations of the substrate dehydroquinone and product dehydroshikimate are very different (Scheme 1). The possibility arises that DHQase enzymes may have to undergo conformational changes to accommodate these alterations in ligand structure. Previous work on the type I enzyme has suggested that the carbocyclic ring of dehydroquinone may be distorted at the active site [44], and recent work has shown that a ligand-induced conformational change can indeed be identified in this class of DHQase [19]. We therefore addressed the question

of whether a ligand-induced conformational change occurs in type II enzymes.

Krell et al. [25] have demonstrated that type II DHQases from both *Streptomyces coelicolor* and *A. nidulans* are inactivated by the arginine-specific reagent PGA. Arg-23 in *S. coelicolor* was determined as the likely site of modification, equivalent to Arg-19 in *A. nidulans*. With both enzymes two types of modification were seen, one resulting in a singly modified arginine residue (Arg-19) and one resulting in the classic Takahashi adduct of two PGA groups covalently attached to a single arginine [45]. The latter residue was not identified. By following the rate of inactivation with the rate of appearance of these adducts, the authors deduced that Arg-19 was the principal site of modification and hence responsible for the loss of activity. Arg-19 is a conserved residue in the type II DHQase family. What role this residue may play in the mechanism remains unclear but, since the present work on the *A. nidulans* enzyme shows that it is very unlikely to be a metal-dependent dehydratase, the possibility arises that Arg-19 may be involved in stabilizing the putative carbanion intermediate. If this were the case and Arg-19 were indeed an active-site residue then it might be expected that ligand binding should affect its modification. This issue was not addressed by Krell et al. [25] in their investigation.

We have reinvestigated the modification of *A. nidulans* type II DHQase by PGA to tackle specific issues not addressed by Krell et al. [25] in their study of the *A. nidulans* enzyme, i.e. the effects of pH and ligand binding, both of which affect the modification by PGA and reveal novel features of the enzyme mechanism. This work also led us to examine the proteolytic susceptibility of the enzyme.

(i) pH effects

Type II DHQases are particularly susceptible to inhibition by a number of buffer systems [8]. To investigate the pH-dependence of PGA-mediated inactivation, we chose to use Hepes because it buffers over a fairly wide range of pH values (6.8–8.2), and the kinetic parameters for the *A. nidulans* enzyme are not dramatically different from those measured in Tris-based buffers at pH 7.0 (k_{cat} 655 s⁻¹; K_m 200 μ M in Hepes). The effect of pH on the inactivation of type II DHQase from *A. nidulans* by PGA is shown in Figure 2(A). Some measurements (notably at pH 9.4) are appreciably outside the buffering range for Hepes; nevertheless, the PGA inactivation data under these conditions compare favourably with those reported by Krell et al. [25]: the measured bimolecular rate constant in Hepes at pH 9.4 is 110 M⁻¹·min⁻¹, compared with 150 M⁻¹·min⁻¹ in bicarbonate buffer. At this pH it is clear that the inactivation follows simple pseudo-first-order kinetics. At lower pH values, however, the kinetics of inactivation become biphasic and this is most noticeable at pH 7.5. It is apparent from these data that two populations of arginine residues are modified (hereby referred to as k_{fast} and k_{slow}) and both seem to result in inactivation of the enzyme. We observed similar biphasic inactivation kinetics with 2,3-butanedione, another arginine-specific reagent [39].

The PGA-concentration-dependence of the biphasic traces showed that both k_{fast} and k_{slow} exhibit pseudo-first-order kinetics and increase with increasing reagent concentration (results not shown). A convenient method for analysing inactivation data is that described by Meloche [46], who derived a linear rate expression that relates the rate of inactivation to the inhibitor concentration. Specifically, the method allows the distinction of simple bimolecular kinetics and affinity labelling, wherein the chemical modification reagent (I) forms a dissociable complex

Figure 2 Kinetics of PGA modification of type II DHQase from *A. nidulans*

(A) Effect of pH on the inactivation by PGA. Enzyme (0.05 mg/ml) was incubated with the reagent (5 mM) at 25 °C in Hepes buffer at pH 7.0 (■), pH 7.5 (□), pH 8.0 (●), pH 8.5 (○) and pH 9.4 (▲), and the residual activity was measured as described in the Materials and methods section. Controls run at each pH indicated that there was no loss of activity in the absence of PGA during these time courses. (B) Concentration-dependence of PGA inactivation kinetics. Replot of the inactivation half-life data of type II DHQase activity over a range of PGA concentrations (0–10 mM) for k_{fast} (●) and k_{slow} (○) in Hepes buffer at pH 7.5 and 25 °C. Inactivation half-lives were taken from semi-log plots of the original data. (C) Effect of ligand binding on the rates of inactivation by PGA. Semi-log plot showing the effect of incubating PGA (5 mM) with enzyme (0.08 mg/ml) in the presence (○) and absence (●) of an equilibrium mixture (1 mM) of substrate and product (molar ratio 1:15; [19]) in Hepes buffer at pH 7.5 and 25 °C. S.E.M. values were obtained from duplicate samples that were assayed at every time point.

with the enzyme (E) prior to inactivation, such as in the kinetic scheme:

Thus the rate of inactivation will reach a limiting value at high (saturating) concentrations of reagent. This can readily be appreciated by replotting the data according to eqn. (1):

$$\tau_{1/2} = 1/[PGA](t_{\min} \cdot K_I) + t_{\min} \quad (1)$$

where $\tau_{1/2}$ is the half-life of inactivation, K_I is $k_{-1} + k_2/k_1$ and $t_{\min}$ is the minimum inactivation half-life at infinite inhibitor concentration. A plot of $\tau_{1/2}$ against $1/[PGA]$ will intercept the y-axis at zero if the reaction is a simple bimolecular process and at a finite value if a reversible enzyme-inhibitor complex is formed prior to inactivation. A replot of $\tau_{1/2}$ against the reciprocal of $[PGA]$ shows that the arginine residue(s) identified as k_{slow} is modified through simple bimolecular kinetics, whereas the residue(s) identified as k_{fast} is modified in a manner consistent with affinity labelling (Figure 2B). The parameters K_I and $t_{\min}$ were determined for k_{fast} for several batches of PGA and were found to vary from batch to batch, probably reflecting the instability of the reagent. The range of values that we measured for K_I and $t_{\min}$ were 6–12 mM and 0.1–0.3 min⁻¹ respectively. Although unusual, affinity labelling by PGA has been observed in other systems [46].

(ii) Effects of ligand binding

Since DHQase is a single-substrate enzyme, protection experiments can only be conducted using an equilibrium mixture of substrate and product [21]. In previous chemical modification experiments using a variety of group-specific reagents with the type I enzyme, such mixtures have shown significant protection against inactivation [20,21]. Hence the effect of a 1 mM substrate/product equilibrium mixture on the inactivation of the type II enzyme by PGA was investigated at pH 7.5 (Figure 2C). It is clear from these data that the substrate/product mixture accelerates k_{fast} but has a negligible effect on k_{slow} ; the bimolecular rate constants for k_{fast} in the absence and presence of this mixture are 9.6 and 13.9 M⁻¹·min⁻¹ respectively, whereas k_{slow} remains at ~ 7.0 M⁻¹·min⁻¹. Thus ligand binding accelerates the rate of inactivation by PGA but at only one of the inactivation sites. Further experiments also showed that the acceleration of k_{fast} by the substrate/product mixture was concentration dependent, being saturated at concentrations > 500 μM [39].

(iii) Stoichiometry of inactivation

It is generally assumed that the stoichiometry of the reaction between PGA and arginine is 2:1, based on observations made with the free amino acid [45]. There are examples, however, of arginine residues in proteins reacting with a stoichiometry of 1:1 [47–49]. In order to investigate the stoichiometry of modification of type II DHQase by PGA at pH 7.5 we analysed the incorporation of radioactivity following incubation of the enzyme in Hepes buffer (50 mM, pH 7.5) at 25 °C with [7-¹⁴C]PGA (see the Materials and methods section) in both the presence and absence of the substrate/product equilibrium mixture (Figure 3). Complete inactivation of the enzyme resulted in ~ 3 mol of PGA incorporated per mol of enzyme subunit, and this stoichiometry remained unchanged on the inclusion of the substrate/product equilibrium mixture.

Figure 3 Stoichiometry of PGA modification of type II DHQase

Enzyme (0.8 mg/ml) in the presence (○) or absence (●) of a substrate/product equilibrium mixture (1 mM) was incubated with [7-¹⁴C]PGA ([¹⁴C]-PG; specific radioactivity 1.85 μCi/μmol; 5.4 mM) in Hepes buffer (50 mM, pH 7.5) at 25 °C. During the time course, samples were removed for measurement of both residual enzymic activity and radioactive incorporation, as described in the Materials and methods section.

(iv) Characterizing the adducts of k_{fast} and k_{slow}

Two approaches were adopted in the characterization of the PGA-derived adducts of type II DHQase, both involving the use of ESI-MS. The first set of experiments examined the derivatives that could be identified and their kinetic profiles in the presence and absence of ligand. The second set of experiments then aimed to identify, through a combination of proteolytic digestion and MS, the arginine residue(s) that was the site of affinity labelling by PGA, the modification of which was enhanced by ligand binding.

The first step in characterizing the adducts of PGA-mediated inactivation of *A. nidulans* type II DHQase involved determining the native subunit molecular mass by ESI-MS. From six independent purifications the observed mass of the protein was 16505 ± 3 Da. Comparing this value with that expected from the published sequence (16517.8 Da [33]) shows that the measured mass is 12 Da lower. The reason for this discrepancy is unknown but it did not preclude the characterization of the PGA adducts.

Inactivation of type II DHQase with 10 mM PGA over 1 h (< 5% residual activity) resulted in the loss of most of the native subunit mass and the appearance of three major adducts at $M + 117$ Da, $M + 249$ Da and $M + 368$ Da (Figure 4A). No other adducts were observed in these experiments. Within the S.E.M. of these measurements (± 2–4 Da), the adduct at $M + 117$ Da is consistent with the covalent attachment of one PGA molecule to a single arginine residue with the concomitant loss of water (expected mass $M + 116$ Da), while the adduct at $M + 249$ Da is consistent with the expected derivative associated with PGA modification in which two molecules are covalently attached to a single arginine residue with the loss of water (expected mass $M + 250$ Da). The final peak at $M + 368$ Da is simply enzyme in which both types of modification have occurred (expected mass $M + 366$ Da). These data are fully in accord with the chemistry of PGA-mediated arginine modification for this enzyme highlighted by Krell et al. [25], in which the initial modification of an arginine residue by a single molecule of PGA to form a *cis* diol can be followed either by rapid dehydration to form a Schiff base (single adduct) species or by further rapid modification to generate the classic Takahashi 2:1 adduct which is more commonly associated

Figure 4 Kinetics of PGA adduct formation monitored by ESI-MS

(A) Electrospray mass spectrum of type II DHQase inactivated to 5% residual activity with PGA (10 mM for 1 h) in Hepes buffer (50 mM, pH 7.5) at 25 °C. The sample was separated from excess reagent by gel-filtration chromatography in Tris buffer and analysed by ESI-MS as described in the Materials and methods section. (B) Kinetics of adduct formation during PGA inactivation of type II DHQase in Hepes buffer (50 mM, pH 7.5). In these experiments the concentration of the reagent was 5 mM and each sample was fractionated by gel filtration at specified time points prior to analysis by ESI-MS. Also plotted are the data showing the rate of enzyme inactivation during this time course.

with PGA-mediated modification of arginine residues. Implicit in such a reaction mechanism is the expectation that the initial modification is sufficient to cause inactivation, since in the many examples of enzymes that show only the Takahashi adduct, simple first-order kinetics are usually observed [50]. What remains unclear, however, is whether the singly modified species represents a kinetically relevant intermediate in the formation of the Takahashi adduct. It has also been pointed out that modification towards the Takahashi adduct could occur through modification of guanidino groups by dimers of PGA [45]. Examples of 1:1 and 2:1 stoichiometries have been observed in other systems (see above), but it is unusual to observe both types of modification in a single enzyme, although this may become more common with the routine use of ESI-MS in characterizing the products of PGA inactivations.

The ESI-MS kinetic profile for the PGA modification of type II DHQase at pH 7.5 shown in Figure 4(B) is compatible with both the kinetic and stoichiometry data presented in Figures 2 and 3. Several points are apparent from these time-course data. (1) The time-dependent loss of the native subunit molecular mass on incubation with PGA follows the loss of DHQase activity

very closely. (2) The dominant adduct, and the one that occurs early in the time course, is the $M+117$ Da peak corresponding to a single addition of PGA. The appearance of the doubly labelled arginine residue ($M+249$ Da) is much slower. (3) The inactivation rate data (Figure 2) show clearly that two populations of arginine residues are modified, the modification of each occurring independently of the other since they display very different labelling behaviour: k_{fast} (the more reactive of the two arginine residues) shows affinity-labelling kinetics, whereas k_{slow} displays simple bimolecular kinetics. The kinetics of these modifications are consistent with the appearance of the $M+117$ and $M+249$ Da peaks, where k_{fast} represents the initial product at $M+117$ Da and k_{slow} the $M+249$ Da peak. Moreover, significant amounts of both the $M+117$ Da and $M+249$ Da peaks occur before the appearance of the $M+368$ Da peak, which is consistent with the modifications representing independent inactivation events on the enzyme. (4) The stoichiometry of PGA modification (Figure 3) was ~ 3 mol of PGA/mol of DHQase monomer, and in separate experiments amino acid analysis showed the loss of one or two arginine residues following complete inactivation by PGA [39]. (5) When the substrate/product equilibrium mixture was present, the amount of the $M+117$ Da peak was enhanced ~ 2 -fold whereas the $M+249$ Da peak remained unaffected (results not shown), which is again consistent with the independent nature of these modifications and the conclusion that the $M+117$ Da peak represents k_{fast} . This latter result also suggests that this residue, while being close to or in the active site, is unlikely to be a central component of the mechanism (e.g. acting as an anion recognition site), since the addition of ligand did not inhibit its modification but enhanced it. We interpret this observation as suggesting that a conformational change occurs on ligand binding that further exposes this arginine to modification, and this results in faster modification and inactivation of the enzyme.

(v) Proteolysis experiments

(a) Identification of k_{fast} . Since the modification of the k_{fast} arginine residue by PGA leads to inactivation of the enzyme through affinity-labelling kinetics (Figure 2) and is enhanced by ligand binding, only this modification was characterized further. Characterizing the adducts of PGA-mediated modification of protein arginine residues is known to be difficult due to the inherent instability of the adducts at neutral pH [45]. While it is certainly feasible to isolate peptides labelled in this way and subject them to N-terminal sequence analysis [50] it is nevertheless problematic, and so we chose to use ESI-MS to characterize the k_{fast} adduct since it required fewer manipulations and provided an unambiguous identification of this residue.

The k_{fast} arginine residue was identified by comparing the products of proteinase K digests of PGA-modified and unmodified type II DHQase (see the Materials and methods section). We found that limited digestion with this protease over ~ 1 h yielded relatively few digest products (around six or seven peptides ranging from 2 to 10 kDa, as deduced from Tricine SDS/PAGE), one of which could be used to assign the labelling position by ESI-MS without recourse to separating the fragments by HPLC. Following either a 30 or 50 min digestion of unmodified type II DHQase with proteinase K, ESI-MS identified a prominent peak at 2736.4 Da which corresponds exactly to the molecular mass of the N-terminal 24 amino acids of the enzyme (calculated mass 2736.2 Da). The sequence of this fragment is MEKSIILLINGPNLNLLGTREPHIY. The proteolytic digestion occurs at Tyr-24–Gly-25. Enzyme that had been inactivated by PGA ($\sim 30\%$ and $\sim 1\%$ activity remaining in two separate

Figure 5 Proteolysis of type II DHQase

(A) Semi-log plot showing the loss of type II DHQase activity (2 mg/ml) on incubation with proteinase K (protein/proteinase ratio 500:1, w/w) in Tris/HCl buffer (50 mM, pH 8.5) at 37 °C in the presence (○) and absence (●) of a 1 mM substrate/product equilibrium mixture. (B) Tricine SDS/PAGE (20%) of type II DHQase following limited digestion with different proteases (see the Materials and methods section). Lane 1, untreated *A. nidulans* type II DHQase; lane 2, chymotrypsin-digested enzyme (25:1; 6 h); lane 3, thermolysin-digested enzyme (25:1; 6 h); lane 4, proteinase K-digested enzyme (500:1; 1 h); lane 5, papain-digested enzyme (25:1; 6 h). Bands of similar apparent molecular mass (indicated by ■) were blotted on to a PVDF membrane and subjected to N-terminal sequencing.

experiments), digested with proteinase K (see the Materials and methods section) and then analysed by ESI-MS showed a decrease in the peak at 2736 Da with a concomitant increase in a new peak at 2851.27 Da, a mass increase of 115 Da. Within the experimental error of these observations (2–4 Da) this is consistent with this peptide as the site of the single modification shown in Figure 4. The question arises, however, as to whether the site of modification is Arg-19 or Lys-3, since PGA is known to form Schiff bases with the ϵ -amino group of lysine residues as well as guanidino groups [44]. Two observations discount Lys-3 as the site of labelling. Firstly, PGA modifies arginine residues significantly more readily than lysine residues and, secondly, amino acid analysis of borohydride-treated PGA-inactivated type II DHQase consistently showed the loss of one or two arginine residues but no loss of lysine [39]. If Lys-3 were being modified by PGA then treatment with borohydride would stabilize the Schiff base, resulting in the loss of a single lysine residue in amino acid analysis hydrolysates.

Arg-19 has previously been identified by Krell et al. as the site of PGA single-adduct formation in both *Streptomyces coelicolor* and *A. nidulans* type II DHQases [25]. However, the present work shows that, at pH 7.5, the modification of Arg-19 by PGA displays affinity-labelling kinetics and is responsive to ligand binding, the latter observation implying that a ligand-induced conformational change occurs on the enzyme.

(b) Proteolytic susceptibility of type II DHQase. During the course of this investigation a number of proteases were analysed for their ability to digest type II DHQase from *A. nidulans*. It proved difficult to digest the enzyme to the level of small peptides with a number of the proteases examined. However, we found

that several enzymes, in particular proteinase K and thermolysin, readily inactivated the enzyme without degrading it to an appreciable extent. The data in Figure 5(A) show that a protein/proteinase K ratio as low as 500:1 (w/w) inactivates type II DHQase over a 60 min time course, exhibiting pseudo-first-order kinetics ($k_{\text{inact}} = 0.07 \text{ min}^{-1}$). The inclusion of a substrate/product equilibrium mixture (1 mM) provided reasonable protection against protease inactivation ($k_{\text{inact}} = 0.04 \text{ min}^{-1}$). Similar data were obtained for thermolysin-mediated inactivation of the enzyme ($k_{\text{inact}} = 0.02 \text{ min}^{-1}$ in the absence of ligand and $k_{\text{inact}} = 0.01 \text{ min}^{-1}$ in the presence of ligand).

The extent of digestion of the proteinase K-inactivated enzyme (10% activity remaining) was analysed under both native and denatured conditions. Calibrated gel-filtration chromatography (see the Materials and methods section) showed that the dodecameric structure of the enzyme remained essentially intact; the inactivated enzyme was eluted between the standards catalase (232 kDa) and yeast alcohol dehydrogenase (151 kDa) (results not shown). Subsequent analysis by both ESI-MS and Tricine SDS/PAGE of the number of cleavage sites showed that six or seven cleavages had in fact occurred over this time course, but these did not disrupt the quaternary structure of the enzyme (Figure 5B). Furthermore, ESI-MS was used to follow the appearance of the N-terminal fragment (2736 Da) which encompasses Arg-19. This fragment was produced within the first few minutes of proteolysis and the amount increased during the time course. Inclusion of ligand decreased by ~40% (after a 40 min incubation with proteinase K) the intensity of this peak (results not shown).

Tricine SDS/PAGE was used to monitor proteolysis of type II DHQase from *A. nidulans* by a number of proteases, including proteinase K and thermolysin (Figure 5B). We noticed that all the proteases tested seemed to produce a similar sized fragment at ~2.5–3.5 kDa. Therefore the peptides for each digest were blotted on to a PVDF membrane (see the Materials and methods section) and these similar sized fragments N-terminally sequenced. In every case, 5–10 rounds of sequencing confirmed that these fragments were the N-terminus of the enzyme.

Conclusions

Type II DHQase from *A. nidulans* is unlikely to be a metal-dependent dehydratase. Stabilization of the carbanion intermediate that is thought to occur following abstraction of the *pro-S* proton may be aided by Arg-19, although binding of ligand accelerates the modification of this residue by PGA at pH 7.5 rather than protecting it, as might be expected for an active-site residue. It appears likely, therefore, that ligand binding causes a conformational change at the N-terminus that further exposes this residue to modification by PGA. The results suggest that Arg-19 may not play a central role in the mechanism but is likely to lie close to the active site. The detected conformational change partially protects the enzyme against proteolysis by a number of proteases, all of which cleave at an exposed site near the N-terminus, one of the most conserved regions in this class of enzyme.

We gratefully acknowledge Dr. David Campbell (University of Dundee, U.K.) for amino acid analysis and N-terminal sequencing of peptides, Dr. Linda Smith for help with CD measurements at the Institute for Food Research (Norwich, U.K.), Mr. Rick Evans-Gowing for technical assistance with the electron microscopy and ESI-MS experiments, and Dr. Mark Harrison (Finnegan MAT, Hemel Hempstead, U.K.) for early ESI-MS data. The Norwich Research Park ESI-MS facility is funded by the Biotechnology and Biological Sciences Research Council. This work was funded by the Agricultural and Food Research Council and the Biotechnology and Biological Sciences Research Council.

REFERENCES

- 1 Haslam, E. (1974) *The Shikimate Pathway*, Butterworths, London
- 2 Bentley, R. (1990) *Biochem. Mol. Biol. Crit. Rev.* **25**, 307–384
- 3 Dewick, P. M. (1993) *Nat. Prod. Rep.* **10**, 233–263
- 4 Hautala, J. A., Jacobson, J. W., Case, M. E. and Giles, N. H. (1975) *J. Biol. Chem.* **250**, 6008–6014
- 5 Hawkins, A. R., Giles, H. H. and Kinghorn, J. R. (1982) *Biochem. Genet.* **20**, 271–286
- 6 Euverink, G. J. W., Hessels, G. I., Vrijbloed, J. W., Coggins, J. R. and Dijkhuizen, L. (1992) *J. Gen. Microbiol.* **138**, 2449–2457
- 7 Kishore, G. M. and Shah, D. M. (1988) *Annu. Rev. Biochem.* **57**, 627–663
- 8 Kleanthous, C., Deka, R., Davis, K., Kelly, S. M., Cooper, A., Harding, S. E., Price, N. C., Hawkins, A. R. and Coggins, J. R. (1992) *Biochem. J.* **282**, 687–695
- 9 Duncan, K., Chaudhuri, S., Campbell, M. S. and Coggins, J. R. (1986) *Biochem. J.* **238**, 475–483
- 10 Servos, S., Chatfield, S., Hone, D., Levine, M., Dimitriadis, G., Pickard, D., Dougan, G., Fairweather, N. and Charles, I. G. (1991) *J. Gen. Microbiol.* **137**, 147–152
- 11 Elsemore, D. A. and Ornston, L. N. (1995) *J. Bacteriol.* **177**, 5971–5978
- 12 Giles, N. H., Partridge, C. W. H., Ahmed, S. I. and Case, M. E. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **58**, 1930–1937
- 13 White, P. J., Young, I., Hunter, I. S., Nimmo, H. G. and Coggins, J. R. (1990) *Biochem. J.* **265**, 735–738
- 14 Garbe, T., Servos, S., Hawkins, A. R., Dimitriadis, G., Young, D. and Charles, I. G. (1991) *Mol. Gen. Genet.* **228**, 385–392
- 15 Lalonde, G., O'Hanley, P. D., Stocker, B. A. D. and Denich, K. T. (1994) *Mol. Microbiol.* **11**, 273–280
- 16 Chaudhuri, S., Duncan, K., Graham, L. D. and Coggins, J. R. (1991) *Biochem. J.* **275**, 1–6
- 17 Hanson, K. R. and Rose, I. A. (1963) *Proc. Natl. Acad. Sci. U.S.A.* **50**, 981–988
- 18 Walsh, C. (1979) *Enzymatic Reaction Mechanisms*, Freeman, San Francisco
- 19 Leech, A. P., James, R., Coggins, J. R. and Kleanthous, C. (1995) *J. Biol. Chem.* **270**, 25827–25836
- 20 Deka, R., Kleanthous, C. and Coggins, J. R. (1992) *J. Biol. Chem.* **267**, 22237–22242
- 21 Kleanthous, C., Campbell, D. G. and Coggins, J. R. (1990) *J. Biol. Chem.* **265**, 10929–10934
- 22 Kleanthous, C. and Coggins, J. R. (1990) *J. Biol. Chem.* **265**, 10935–10939
- 23 Shneier, A., Harris, J., Kleanthous, C., Coggins, J. R., Hawkins, A. R. and Abell, C. A. (1993) *Bioorg. Med. Chem. Lett.* **3**, 1399–1402
- 24 Harris, J., Kleanthous, C., Coggins, J. R., Hawkins, A. R. and Abell, C. (1993) *J. Chem. Soc. Chem. Commun.* 1080–1081
- 25 Krell, T., Pitt, A. R. and Coggins, J. R. (1995) *FEBS Lett.* **360**, 93–96
- 26 Hawkins, A. R. (1987) *Curr. Genet.* **11**, 491–498
- 27 Grazi, E., Cheng, T. and Horecker, B. L. (1962) *Biochem. Biophys. Res. Commun.* **7**, 250–253
- 28 Horeker, B. L., Tsolas, O. and Lai, C. Y. (1972) *Enzymes 3rd Ed.* **7**, 213–258
- 29 Creighton, D. J. and Murthy, N. S. R. K. (1990) *Enzymes, 3rd Ed.* **24**, 323–421
- 30 Grewe, R. and Haendler, H. (1968) *Biochem. Prep.* **12**, 21–26
- 31 Beri, R. K., Grant, S., Roberts, C. F., Smith, M. and Hawkins, A. R. (1990) *Biochem. J.* **265**, 337–342
- 32 Hawkins, A. R. and Smith, M. (1991) *Eur. J. Biochem.* **196**, 717–724
- 33 Da Silva, A. J. F., Whittington, H., Clements, J., Roberts, C. and Hawkins, A. R. (1986) *Biochem. J.* **240**, 481–488
- 34 Auld, D. S. (1988) *Methods Enzymol.* **158**, 13–14
- 35 Wolnik, K. A. (1988) *Methods Enzymol.* **158**, 190–205
- 36 Harris, J. R. (1992) *Microsc. Anal.* **30**, 13–16
- 37 Kleanthous, C., Cullis, P. M. and Shaw, W. V. (1985) *Biochemistry* **24**, 5307–5313
- 38 Schagger, H. and Von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379
- 39 Bottomley, J. R. (1995) Ph.D. Thesis, University of East Anglia, Norwich, U.K.
- 40 Auld, D. S. (1988) *Methods Enzymol.* **158**, 110–114
- 41 Vallee, B. L. and Galde, A. (1984) *Adv. Enzymol. Relat. Areas Mol. Biol.* **56**, 283–430
- 42 Branden, C. and Tooze, J. (1991) *Introduction to Protein Structure*, Garland, New York
- 43 Valentine, C. R., Shapiro, B. M. and Stadtman, E. R. (1968) *Biochemistry* **7**, 2143–2152
- 44 Vaz, A. D. N., Butler, J. R. and Nugent, M. J. (1975) *J. Am. Chem. Soc.* **97**, 5914–5915
- 45 Takahashi, K. (1968) *J. Biol. Chem.* **243**, 6171–6179
- 46 Meloche, H. P. (1967) *Biochemistry* **6**, 2273–2280
- 47 Phillips, M., Pho, D. B. and Pradel, L.-A. (1979) *Biochim. Biophys. Acta* **566**, 296–304
- 48 Borders, Jr., C. L. and Riorden, J. F. (1975) *Biochemistry* **14**, 4699–4704
- 49 Werber, M. M., Moldovan, M. and Sokolovsky, M. (1975) *Eur. J. Biochem.* **53**, 207–216
- 50 Bond, M. W., Chiu, N. Y. and Cooperman, B. S. (1980) *Biochemistry* **19**, 94–102