Characterization of the type I dehydroquinase from *Salmonella typhi*

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The type I dehydroquinase from the human pathogen *Salmonella typhi* was overexpressed in an *Escherichia coli* host and purified to homogeneity. The *S. typhi* enzyme was characterized in terms of its kinetic parameters, important active-site residues, thermal stability and c.d. and fluorescence properties. In all important respects, the enzyme from *S. typhi* behaves in a very similar fashion to the well-characterized enzyme from *E. coli*, including the remarkable conformational stabilization observed on reduction of the substrate/product mixture by $NaBH_4$. This gives confidence that the information from X-ray studies on the *S. typhi* enzyme [Boys, Fawcett, Sawyer, Moore, Charles, Hawkins, Deka, Kleanthous and Coggins (1992) J. Mol. Biol. **227**, 352–355] can be applied to other type I dehydroquinases. Studies of the quenching of fluorescence of the *S. typhi* enzyme by succinimide show that $NaBH_4$ reduction of the substrate/product imine complex involves a dramatic decrease in the flexibility of the enzyme, with only very minor changes in the overall secondary and tertiary structure.

INTRODUCTION

The enzyme dehydroquinase (3-dehydroquinate dehydratase, EC 4.2.1.10) catalyses the dehydration of 3-dehydroquinic acid to 3-dehydroshikimic acid. This reaction is common to two metabolic pathways: the shikimate pathway for the synthesis of aromatic compounds such as the aromatic amino acids and ubiquinone, and the catabolic quinate pathway of fungi, which allows the organism to utilize quinate as a carbon source via the β-oxoadipate pathway. The shikimate pathway, which is present in micro-organisms and plants, but absent for higher animals, has attracted widespread interest as a target for antimicrobial agents and herbicides (Kishore and Shah, 1988). Shikimate-pathway mutants of *Salmonella typhimurium* have also been shown to be excellent live vaccines (O'Callaghan et al., 1988).

Dehydroquinases fall into two distinct classes (White et al., 1990; Servos et al., 1991; Kleanthous et al., 1992), which are distinguished by non-homologous amino acid sequences (Charles et al., 1986; Da Silva et al., 1986; Duncan et al., 1987; Chaudhuri et al., 1991; Garbe et al., 1991) and biophysical criteria (Kleanthous et al., 1992). They have thus been proposed to have arisen by convergent evolution.

Type I enzymes involve a covalent imine intermediate to catalyse a *cis* elimination (Butler et al., 1974; Shneier et al., 1991). [Addition of substrate (3-dehydroquinate) to active enzyme leads to the formation of an equilibrium mixture of substrate and product (3-dehydroshikimate) ($K_{eq.} = 15$; Kleanthous et al., 1990). This is referred to as the 'substrate/product mixture'. On addition of $NaBH_4$, the imine intermediates involved in the catalytic process are reduced, leading to inactivation of the enzyme. This inactivated enzyme preparation is referred to as the 'ligand-linked enzyme'.] These enzymes are dimers of subunit M_r around 27000. By contrast, type II enzymes do not involve an imine intermediate, catalyse a *trans* elimination and are usually dodecameric of subunit M_r around 16000 (Abell et al., 1993; Kleanthous et al., 1992). The type II dehydroquinases have been shown to catalyse reactions in both the catabolic quinate utilization (Hawkins et al., 1982; Euverink et al., 1992) and biosynthetic shikimate pathways (Garbe et al., 1991; White et al., 1990). By contrast, type I dehydroquinases have only been observed to have roles in the biosynthetic shikimate pathway.

In plants and fungi, dehydroquinase occurs associated with other enzymes of the biosynthetic pathway. In plants, it is fused with shikimate dehydrogenase, the next enzyme in the shikimate pathway, to form a bifunctional enzyme (Koshiba, 1978; Mousdale et al., 1987). In lower eukaryotes, such as *Neurospora crassa*, *Aspergillus nidulans*, *Saccharomyces cerevisiae* and *Euglena gracilis*, the five central steps from 3-deoxy-D-arabinoheptulosonic acid 7-phosphate to 5-enoylpyruvoylshikimate 3-phosphate are catalysed by a single pentafunctional enzyme encoded by the *arom* gene (Lumsden and Coggins, 1977; Patel and Giles, 1979; Charles et al., 1985, 1986; Duncan et al., 1987). In prokaryotes, the five enzymes are encoded by separate genes scattered throughout the genome. Sequence comparisons reveal, however, that there is considerable identity between the *arom* sequence of *A. nidulans* and *S. cerevisiae* and the sequences of the corresponding enzymes from *Escherichia coli*, including conservation of amino acids thought to be important in the catalytic mechanisms (Charles et al., 1985, 1986; Hawkins, 1987; Duncan et al., 1987). Several of the domains of the AROM protein are capable of independent function (Smith and Coggins, 1983; Coggins et al., 1985; Hawkins and Smith, 1991; Moore and Hawkins, 1993) in the absence of the entire pentafunctional polypeptide. It seems very likely that the *arom* genes have arisen by fusion of ancestral genes encoding the monofunctional proteins (Duncan et al., 1987; Hawkins, 1987).

Abbreviations used: DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; GdnHCl, guanidinium chloride; ANS, 8-anilino-1-naphthalenesulphonate; DSC, differential scanning calorimetry.

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In order to understand the pentafunctional enzyme, the corresponding isolated enzymes from prokaryotes have been studied in some detail. Chemical modification of *E. coli* dehydroquinase has demonstrated the involvement of Lys-170 and His-143 in the catalytic mechanism and the role of Met-23 and Met-205 in substrate binding (Kleanthous and Coggins, 1990; Kleanthous et al., 1990; Chaudhuri et al., 1991; Deka et al., 1992). Formation of the ligand-linked enzyme leads to dramatic stabilization against proteolysis, thermal denaturation and unfolding by guanidinium chloride (GdnHCl) (Kleanthous et al., 1991, 1992). The crystals obtained from *E. coli* dehydroquinase form thin laminated plates and are not suitable for structure determination by X-ray crystallography (Boys et al., 1992). Dehydroquinase has been isolated from the important human pathogen *S. typhi*; the enzyme is 69% identical in sequence with the *E. coli* enzyme (Servos et al., 1991; Chaudhuri et al., 1991) and gives crystals which diffract to a resolution of 0.23 nm (Boys et al., 1992). Structural studies of the *S. typhi* enzyme (which has been overproduced to approx. 50% of total cell protein in *E. coli*; Moore et al., 1992) in its native and ligand-linked form are currently underway.

This paper reports the purification to homogeneity of the type I dehydroquinase from *S. typhi*, and presents data which show that the *S. typhi* enzyme behaves in solution in a very similar fashion to the *E. coli* enzyme with regard to its kinetic properties, including inactivation by diethyl pyrocarbonate (DEPC), its secondary structure and the stabilization afforded by formation of the ligand-linked enzyme. This establishes that the structural information derived from X-ray diffraction of the *S. typhi* enzyme will be generally applicable to the interpretation of the behaviour of the *E. coli* and other type I enzymes. In addition, data from fluorescence quenching offer additional insights into the stabilization of the enzyme afforded by formation of the ligand-linked enzyme.

EXPERIMENTAL

Isolation of *S. typhi* dehydroquinase

The type I dehydroquinase of *S. typhi* was purified from an overproducing strain of *E. coli*. The plasmid pKK45 containing the PCR-amplified coding sequence of the *S. typhi* type I dehydroquinase cloned into the commercially available expression vector pKK233-2 (Pharmacia) was described by Moore et al. (1992).

Step 1: growth, induction and lysis of *S. typhi* dehydroquinase overproducing *E. coli* cells

The dehydroquinase-lacking *aroD* mutant strain of *E. coli*, SK3430 (Kinghorn and Hawkins, 1982), was transformed with pKK45 DNA. Ten 500 ml cultures of mid-exponential-phase [attenuance $(D)_{500} = 0.2$] strain SK3430 harbouring pKK45 incubated at 37 °C were induced in the presence of 200 μg/ml isopropyl β-D-thiogalactopyranoside for 9 h. The cultures were harvested by centrifugation at 2500 *g* at 4 °C for 10 min and the cell paste pellets were pooled and stored at −20 °C until lysed. All subsequent steps were carried out at 4 °C unless otherwise noted. Frozen cell paste (approx. 36 g wet wt.) was thawed and resuspended in 175 ml of extraction buffer [100 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM benzamidine and 1 mM phenylmethanesulphonyl fluoride; buffer A]. The cells were then lysed by sonication in an MSE Soniprep 150 sonic disruptor. Cellular debris was then pelleted by centrifugation at 10000 *g* at 4 °C for 20 min. The supernatant was designated the crude extract.

Step 2: fractionation with $(NH_4)_2SO_4$

The fraction precipitated between 30% and 75% saturation was collected by centrifugation at 4 °C (10000 *g* for 20 min). The precipitate was redissolved in 37 ml of buffer B (50 mM Tris/HCl, pH 7.5, 1 mM DTT) and dialysed at 4 °C overnight against 2 litres of buffer B.

Step 3: anion-exchange chromatography on DEAE-Sephacel

A 15 cm × 16 cm^2 DEAE-Sephacel column was equilibrated with 5 column volumes of buffer B. The dialysed dehydroquinase-containing fraction was loaded on to the column, which was then washed with 2 column volumes of buffer B. Bound proteins were then eluted with a 0–500 mM NaCl linear gradient in 1 litre of buffer B; 10 ml fractions were collected and assayed for dehydroquinase activity. Fractions containing in excess of 500 units of dehydroquinase activity/ml were pooled and dialysed overnight against 2 litres of buffer C (20 mM Tris/HCl, 0.4 mM DTT, pH 7.5).

Step 4: high-resolution anion-exchange chromatography on Neobar AQ[4]

High-performance anion-exchange chromatography was carried out at room temperature on a Pharmacia f.p.l.c. system. A 4 ml Neobar AQ[4] column (Flowgen) had been regenerated with 5 column volumes of buffer C containing 1 M NaCl, and subsequently equilibrated with 5 column volumes of buffer C. The dialysed dehydroquinase-containing pooled fractions from DEAE-Sephacel chromatography [approx. 40 mg of protein as estimated by the method of Bradford (1976) for each run] were loaded on to the Neobar AQ[4] column, which was then washed with 5 volumes of buffer C. Bound proteins were eluted with an 80 ml linear gradient of 0–250 mM NaCl in buffer C; 2 ml fractions were collected. Fractions were analysed by SDS/PAGE (Laemmli, 1970), and those containing apparently homogeneous *S. typhi* dehydroquinase (subunit M_r 28000) were pooled and dialysed against storage buffer [50 mM potassium phosphate buffer, pH 7.0, 1 mM DTT, 50% (v/v) glycerol].

Estimation of the native M_r by size-exclusion chromatography

The native M_r of the enzyme was estimated by size-exclusion chromatography (in 50 mM Tris/HCl, pH 7.5, containing 200 mM 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).

Dehydroquinase assays

The type I dehydroquinase of *S. typhi* was assayed in 50 mM potassium phosphate buffer, pH 7.0, at 25 °C. The substrate 3-dehydroquinate was at a concentration of 100 μM for standard assays during purification, at a concentration of 128 μM for the DEPC inactivation experiments, and was varied from 2 to 80 μM for the kinetic characterization experiments. Kinetics constants were obtained from Hanes plots ([S]/v against [S]) by using linear regression. The errors in the values of $k_{cat.}$ and K_m are estimated to be ±5%. Assays were performed by monitoring the production of 3-dehydroshikimate at 234 nm ($\varepsilon = 1.2 \times 10^4$ M$^{-1}\cdot$cm^{-1}). Experiments for determining the dependence of the activity of

S. typhi dehydroquinase on pH were performed in 50 mM potassium phosphate buffer (pH 6.6–7.9) and 50 mM citrate/phosphate buffer (pH 4.6–6.8) at 25 °C, with dehydroquinate concentrations varying between 8 μM and 512 μM.

Production of ligand-linked type I dehydroquinase

This was performed using $NaBH_4$ as described by Kleanthous et al. (1992), except that the concentration of substrate (dehydroquinate) added was 2 mM.

Kinetics of inactivation of *S. typhi* dehydroquinase by DEPC

The kinetics of inactivation of *S. typhi* dehydroquinase by DEPC were investigated essentially as described by Deka et al. (1992) for the enzyme from *E. coli*. Protection experiments in which the *S. typhi* dehydroquinase was exposed to DEPC in the absence and presence of the substrate/product mixture were also performed. The pH-dependence of the inactivation of *S. typhi* by DEPC was determined in 100 mM potassium phosphate buffer over the pH range 5.7–6.7. The extent of N-carbethoxylation of histidine residues was determined from the increase in the A_{240}, as described by Deka et al. (1992), by using a value of 3200 for the molar absorbance of *N*-carbethoxyhistidine (Ovadi et al., 1967). The *N*-carbethoxylation of histidine side chains was reversed by incubation with 500 mM hydroxylamine in 50 mM potassium phosphate buffer, pH 7.0, for 8 h at 20 °C, followed by dialysis against this buffer to remove the hydroxylamine. Before incubation with hydroxylamine, samples of enzyme which had been quenched with 40 mM imidazole were dialysed against 50 mM potassium phosphate buffer, pH 7.0, to remove the imidazole. The progress of the removal of carbethoxyl groups was monitored by difference spectroscopy and by assays of dehydroquinase activity.

The concentrations of solutions of *S. typhi* dehydroquinase were determined spectrophotometrically by using a value of 0.45 for the A_{278} of a 1 mg/ml solution of enzyme in buffer (50 mM potassium phosphate, pH 7.0) in a cuvette of path-length 1 cm. This value was calculated on the basis of the known tyrosine and tryptophan contents of the protein (Servos et al., 1991) by using the method of Gill and von Hippel (1989), and confirmed by amino acid analysis.

C.d. measurements

C.d. spectra were recorded at 20 °C in a JASCO J-600 spectropolarimeter. Molar ellipticity values were calculated by using a value of 110 for the mean residue weight, derived from the amino acid sequence of the protein (Servos et al., 1991). Protein concentrations were typically 0.1–0.15 mg/ml (0.05 cm path-length) and 0.5–0.8 mg/ml (0.5 cm path-length) for far-u.v. and near-u.v. spectra respectively.

Fluorescence measurements

Fluorescence spectra were recorded at 20 °C in a Perkin–Elmer LS50 spectrofluorimeter. Raman scattering by the solvent was corrected for by using appropriate blank solutions. The quenching of protein fluorescence by succinimide was performed and analysed as described previously (Johnson and Price, 1987). Succinimide and acrylamide were recrystallized from ethanol and ethyl acetate respectively before use. Correction for the inner-filter effect caused by the absorption of incident radiation by acrylamide was performed as described by Ward (1985). The fluorescence of 8-anilino-1-naphthalenesulphonate (ANS) (20 μM) in the absence and presence of protein (0.05 mg/ml) was monitored using excitation and emission wavelength of 380 nm and 470 nm respectively.

GdnHCl (Ultrapure grade) was purchased from Gibco–BRL, Paisley, Scotland, U.K. The concentrations of solutions of GdnHCl were checked by refractive-index measurements (Nozaki, 1972). Denaturation of the enzyme by GdnHCl was monitored by fluorescence and far-u.v. c.d. measurements after incubation of the solutions for 24 h to ensure equilibration (Kleanthous et al., 1991).

Differential scanning calorimetry (DSC)

These experiments were performed over a range of 20–110 °C by using a Microcal MC-2D instrument at a scan rate of 60 °C/h as described by Kleanthous et al. (1991).

RESULTS

Purification and initial kinetic characterization of the *S. typhi* type I dehydroquinase

The type I dehydroquinase from *S. typhi* was purified to apparent homogeneity (see Figure 1) by a simple purification protocol which, if the final step was carried out repeatedly, could yield

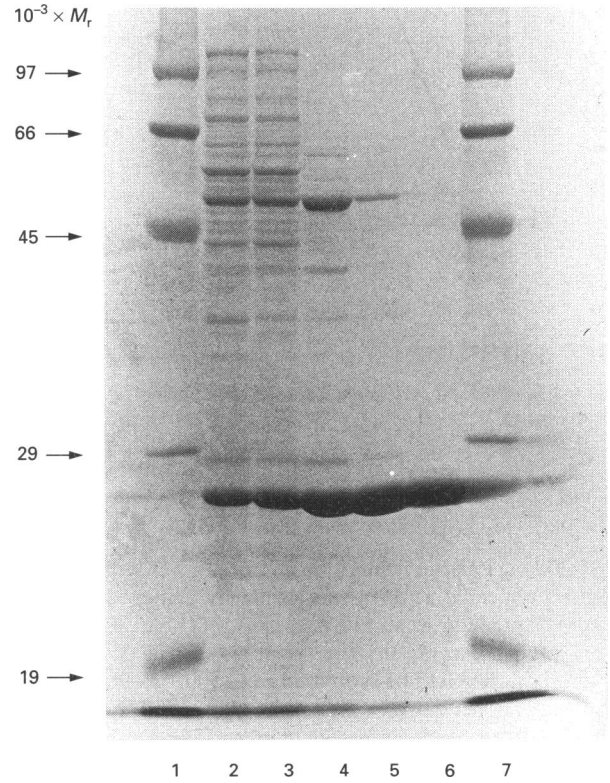

Figure 1 Purification of *S. typhi* dehydroquinase, analysed by SDS/PAGE

Lanes 1 and 7, M_r markers (phosphorylase, 97000; BSA, 66000; ovalbumin, 45000; carbonic anhydrase, 29000; β-lactoglobulin, 19000); lane 2, crude extract; lane 3, proteins precipitated by $(NH_4)_2SO_4$ between 30 and 75% saturation; lane 4, after anion-exchange chromatography on DEAE-Sephacel; lane 5, after high-resolution anion-exchange chromatography on Neobar AQ[4] (150 mg loaded on to column); lane 6, after high-resolution anion-exchange chromatography on Neobar AQ[4] (40 mg loaded on to column). The gel was stained for protein with Coomassie Brilliant Blue.

Table 1 Purification of *S. typhi* type I dehydroquinase

Steps 1, 2, 3 and 4 refer to the crude extract, the 30–75%-satn.-$(NH_4)_2SO_4$ fraction, the DEAE-Sephacel fraction and the Neobar AQ[4] fraction respectively. The initial wet weight of cells was 36 g. One unit of enzyme activity corresponds to 1 μmol of product formed/min at 25 °C.

Step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)
1	160	4000	112000	28	100
2	71	3500	109000	31	97
3	81	1215	121000	100	108
4	100	174	39900	229	36

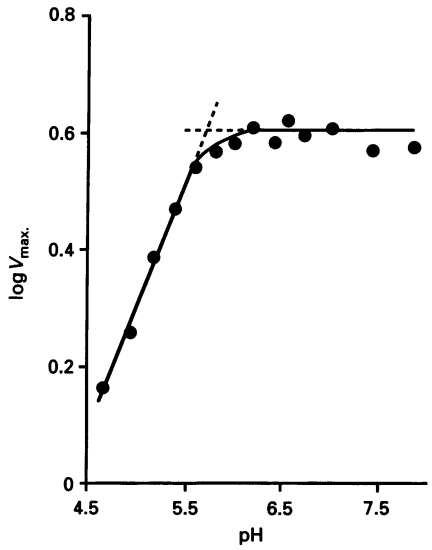

Figure 2 pH-dependence of $V_{max.}$ for *S. typhi* dehydroquinase

The pH was varied over the range 4.5–8 with potassium phosphate and phosphate/citrate buffers as described in the text.

nearly 200 mg of pure protein from the 36 g wet wt. of cells. The increase in specific activity was primarily achieved by the two anion-exchange-chromatography steps (Table 1). By size-exclusion chromatography the native M_r of the *S. typhi* type I dehydroquinase could be estimated as 61500, which is consistent with the enzyme being a dimer of M_r-27700 subunits. The corresponding enzyme of *E. coli* was also found to be dimeric by size-exclusion chromatography (Chaudhuri et al., 1986), sedimentation velocity and equilibrium centrifugation (Kleanthous et al., 1992).

Like the type I dehydroquinases of *E. coli* and *N. crassa* (part of the pentafunctional *arom* protein), the *S. typhi* dehydroquinase was irreversibly inactivated by treatment with $NaBH_4$ in the presence of a substrate/product mixture (results not shown). In the absence of the substrate/product mixture, addition of $NaBH_4$ to the enzyme in solution had no effect on the enzyme activity. These results confirm that, like the type I dehydroquinase of *E. coli*, the *S. typhi* type I dehydroquinase is active as a dimer, and has a mechanism that involves the formation of a covalent imine intermediate between a lysine side chain on the enzyme and the substrate. The importance of this lysine is also indicated by its conservation in all type I enzymes (Kleanthous et al., 1992).

S. typhi dehydroquinase was found to follow Michaelis–Menten kinetics, with a K_m for dehydroquinate of 18 μM and a $k_{cat.}$ of 200 s^{-1}, and thus a $k_{cat.}/K_m$ of 1.1×10^7 $M^{-1} \cdot s^{-1}$ at 25 °C in 50 mM potassium phosphate buffer (pH 7.0). These values are similar to those reported for the *E. coli* enzyme, with a K_m of 16 μM, $k_{cat.}$ of 135 s^{-1} and a $k_{cat.}/K_m$ of 8.4×10^6 $M^{-1} \cdot s^{-1}$ under the same conditions (Chaudhuri et al., 1986; Kleanthous et al., 1992).

A plot of log $V_{max.}$ against pH in the range 4.5–8 (Figure 2) indicates that a single ionizable group with a pK_a estimated as 5.7 is involved in the mechanism. A similarly essential ionizable group in the mechanism of the corresponding enzyme of *E. coli*, with a pK_a of 6.1, was identified by Chaudhuri et al. (1986); more recently Deka et al. (1992) have shown that this ionizable group is His-143, a residue conserved in the sequence of all known type I dehydroquinases.

Kinetics of inactivation of the *S. typhi* dehydroquinase by DEPC

Incubation of the *S. typhi* dehydroquinase with DEPC at 25 °C in 50 mM potassium phosphate buffer (pH 6.0) resulted in the rapid loss of activity, and a plot of the logarithm of remaining activity versus time (corrected for the hydrolysis of DEPC in the buffer; Rakitzis, 1984) at various concentrations of DEPC (Figure 3a) shows that the reaction obeys pseudo-first-order kinetics. The first-order rate constants are proportional to the DEPC concentration (Figure 3b), demonstrating that this inactivation is a bimolecular process which is not dependent on the formation of a reversible enzyme–DEPC complex before inactivation (Church et al., 1985). The second-order rate constant for the inactivation of the *S. typhi* dehydroquinase by DEPC calculated from these data is 150 $M^{-1} \cdot min^{-1}$, essentially identical with the corresponding value reported by Deka et al. (1992) for the inactivation of the *E. coli* dehydroquinase by DEPC (148.5 $M^{-1} \cdot min^{-1}$). Figure 3(c) shows that the prior addition of increasing concentrations of the substrate/product mixture protects against the inactivation of the *S. typhi* dehydroquinase by DEPC, suggesting that the inactivation is active-site-directed. The pK_a of the reactive side chain was calculated to be 5.8 from the pH-dependence of the rate of inactivation by using the procedure of Deka et al. (1992); this value is close to that estimated from the plot of log $V_{max.}$ against pH (Figure 2).

Number of essential histidine residues

Figure 4(a) shows that the inactivation of the *S. typhi* type I dehydroquinase by DEPC is linearly dependent on the number of histidine residues modified, up to approx. 5 histidines modified per subunit. Analysis of the data in this initial part of the modification reaction by the method of Tsou (1962) is consistent with one critical residue being involved in the mechanism (Figure 4b). Similar results had been reported for the corresponding *E. coli* enzyme (Deka et al., 1992). In contrast with the *E. coli* dehydroquinase, which has 6 histidine residues per subunit (Duncan et al., 1986; Chaudhuri et al., 1991), the *S. typhi* type I dehydroquinase contains a total of 9 histidine residues (Servos et al., 1991).

The data in Figure 4(a) show that, once a 'critical' number of histidine residues (about 5 per subunit) have been modified, the A_{240} begins to increase sharply and reaches a value above that corresponding to N-carbethoxylation of the total number of

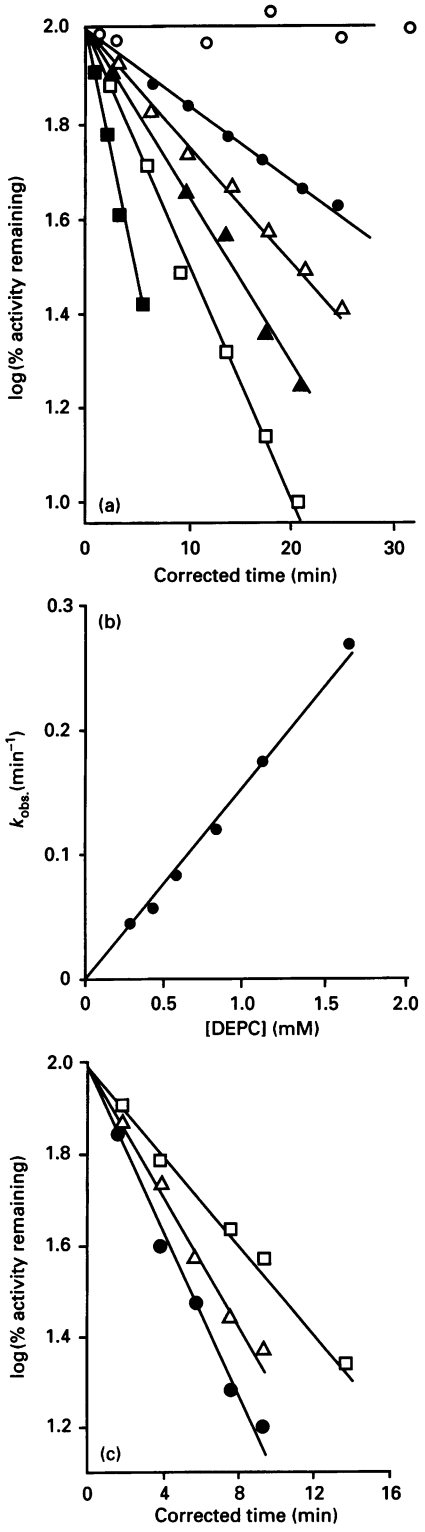

Figure 3 Kinetics of inactivation of *S. typhi* dehydroquinase by DEPC

Enzyme (70 μg/ml) was treated with DEPC in 50 mM potassium phosphate, pH 6.0. (**a**) Semi-logarithmic plots showing the loss of activity at the following concentrations of DEPC: ○, 0 mM; ●, 0.28 mM; △, 0.42 mM; ▲, 0.56 mM; □, 0.83 mM; ■, 1.67 mM. The time axis was corrected for the hydrolysis of DEPC in accordance with Rakitzis (1984), data of Berger (1975) and Miles (1977) being used to estimate k' for the hydrolysis of DEPC as 0.015 min^{-1}. (**b**) The pseudo-first-order rate constants from (**a**) plotted against the concentration of DEPC. (**c**) Protection against inactivation by 1.12 mM DEPC by inclusion of the substrate/product mixture; ●, △ and □ represent initial added dehydroquinate concentrations of 0, 128 μM and 640 μM respectively.

histidines in the enzyme. This phenomenon was observed over a wide range of dehydroquinase and DEPC concentrations. It can be speculated that this phenomenon is caused by unfolding of the modified proteins which may lead to the modification of previously buried histidine side chains as well as tyrosine or lysine side chains, together with the possible N-carbethoxylation of both imidazole nitrogen atoms in the histidine residues (Miles, 1977). The data in Figure 4(a) show that in the case of the ligand-linked enzyme the upward curvature does not occur. (This is also found even after incubation with much higher concentrations of DEPC for long periods.) This suggests that in the case of the ligand-linked enzyme the putative unfolding does not occur, presumably as a result of the conformational stabilization noted below. Figure 4(a) shows that a small number of histidine residues (one or two) is protected against modification in the ligand-linked enzyme.

Incubation of the DEPC-modified enzyme with hydroxylamine led to significant levels of re-activation. After incubation of 10 μM dehydroquinase with 0.5 mM DEPC for periods of 5, 15, 30 and 60 min, the activity remaining was 70%, 53%, 45% and 1% of the control respectively. Treatment of these samples with hydroxylamine increased the activity to 90%, 86%, 81% and 2% of the respective control samples. From these results it is clear that the inactivation in at least the initial phase of the reaction is due to N-carbethoxylation of histidines. However, at later stages of the reaction with DEPC, irreversible inactivation has occurred, as a result of unfolding of the enzyme or additional chemical modification. The lack of complete re-activation by hydroxylamine has been noted in the studies on the *E. coli* dehydroquinase (Deka et al., 1992).

Spectroscopic properties of *S. typhi* dehydroquinase

C.d. spectra

The far-u.v. and near-u.v. c.d. spectra of native and ligand-linked dehydroquinase from *S. typhi* are shown in Figures 5(a) and 5(b) respectively. The spectra are closely comparable with those reported previously for the two forms of the enzyme from *E. coli* (Kleanthous et al., 1991). The far-u.v. c.d. spectra show that modification of the *S. typhi* enzyme causes little change in the secondary structure, as was found for the *E. coli* enzyme (Kleanthous et al., 1991). As determined by the CONTIN procedure (Provencher and Glöckner, 1981), the secondary-structure contents for the native and ligand-linked enzymes are: α-helix, 39% and 41%; β-sheet, 28 and 24%; remainder, 33% and 35% respectively. These values are very similar to those reported for the *E. coli* enzyme (Kleanthous et al., 1991); the small differences probably arise from small errors in the determination of protein concentrations.

The near-u.v. c.d. spectra show small differences between the native and ligand-linked forms of the *S. typhi* enzyme (as also observed for the *E. coli* enzyme; Kleanthous et al., 1991), suggesting that some small differences in tertiary structure may occur on formation of the ligand-linked derivative.

Fluorescence spectra

When excited at 290 nm, native and ligand-linked dehydroquinase show emission maxima at 329 nm and 328 nm respectively (Figure 5c), indicating in each case that the single tryptophan side chain has only a moderate degree of exposure to the solvent (Eftink and Ghiron, 1976). The small differences in fluorescence intensity (that of the ligand-linked form is about 10% lower than the native form) and in the emission maximum of the two forms of the enzyme indicate small differences in the

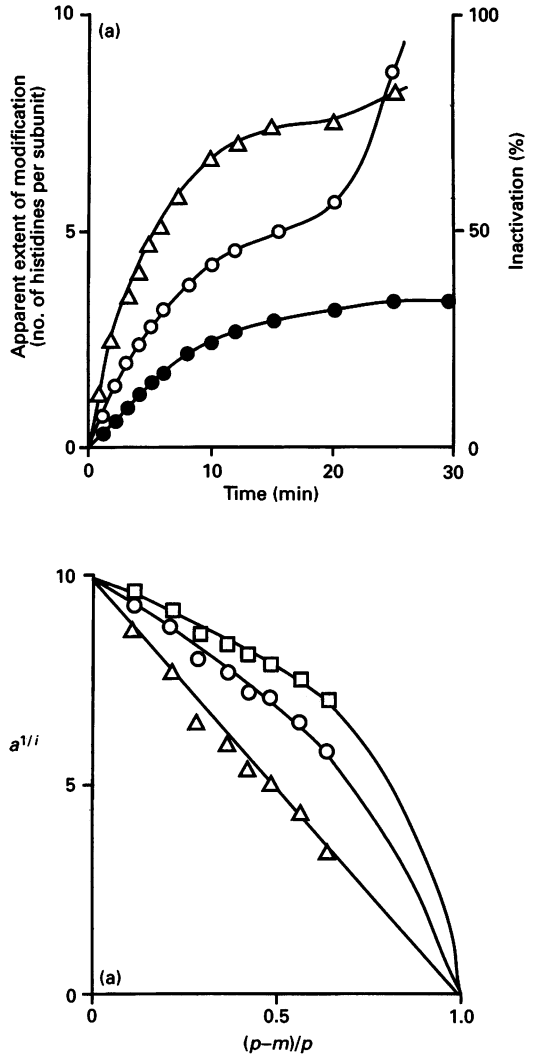

Figure 4 Stoichiometry of modification of *S. typhi* dehydroquinase by DEPC

(**a**) Extent of modification of native (○) and ligand-linked (●) enzyme (0.14 mg/ml) by DEPC (0.42 mM) monitored by changes in A_{240}; (△) shows the extent of inactivation of the native enzyme by DEPC. (**b**) Plot of the activity data for native enzyme from (**a**) in accordance with Tsou (1962), 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 residue(s) critical for activity, and p is the total number of groups modified in the first phase of the reaction, i.e. before the upward curvature of the plot of A_{240} against time. This number is estimated to be 6 from the progress curve shown in (**a**). △, ○ and □ represent i values of 1, 2 and 3 respectively.

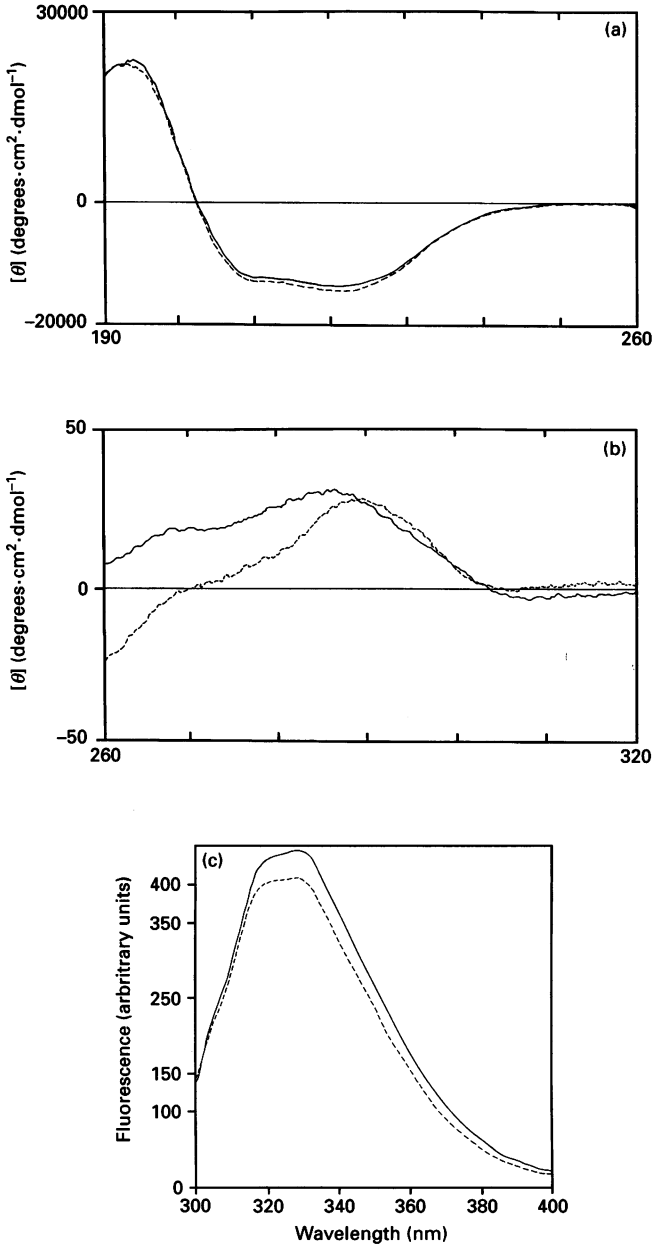

Figure 5 Spectroscopic properties of *S. typhi* dehydroquinase

(**a**) Far-u.v. c.d. spectra; (**b**) near-u.v. c.d. spectra; (**c**) fluorescence spectra: —— and ---- represent native and ligand-linked enzymes respectively. Spectra were recorded in 50 mM potassium phosphate buffer, pH 7.0, at 20 °C.

tertiary structures, consistent with the near-u.v. c.d. data (Figure 5b).

Unfolding of native and ligand-linked dehydroquinase by GdnHCl

Spectroscopic measurements

On addition of 6 M GdnHCl, both forms of the enzyme are completely unfolded, as indicated by the far-u.v. c.d. spectrum, which is typical of a random coil with the ellipticity at 225 nm increased to < 10% of the value for the native enzymes, and by the fluorescence emission maximum, which is shifted to 355 nm, a value typical of fully exposed tryptophan side chains (Eftink and Ghiron, 1976).

The changes in the ellipticity at 225 nm and in the fluorescence at 330 nm of both native and ligand-linked enzyme at increasing concentrations of GdnHCl are shown in Figure 6. The ordinate shows the changes expressed as a percentage of the maximum change observed between 0 and 6 M GdnHCl. It is clear that formation of the ligand-linked enzyme leads to a dramatic stabilization; the mid-points of the changes in ellipticity at 225 nm (reflecting loss of secondary structure) occur at GdnHCl concentrations of 1.6 M and 4.5 M for the native and ligand-

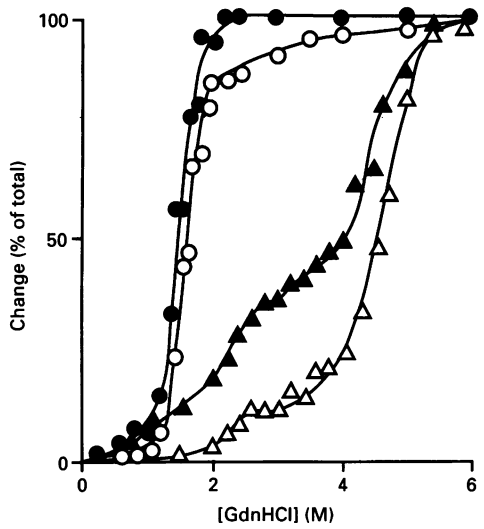

Figure 6 Unfolding of *S. typhi* dehydroquinase by GdnHCl

The structural changes were monitored by far-u.v. c.d. (○, △) and fluorescence (●, ▲). Data for native and ligand-linked enzymes are shown as (○, ●) and (△, ▲) respectively. In each case the changes are shown relative to the total change in the particular parameter observed between 0 and 6 M GdnHCl. Experiments were performed at enzyme concentrations of 0.1 mg/ml in potassium phosphate buffer, pH 7.0, at 20 °C.

Table 2 Stern–Volmer constants for quenching by succinimide

The Stern–Volmer constant (K_{SV}) were estimated from measurements of the fluorescence of samples (0.1 mg/ml) at 330 nm (20 °C) on addition of portions of succinimide. The range of succinimide concentrations used was 0–300 mM, over which the Stern–Volmer plots were linear.

Sample	K_{SV} (M^{-1})
Native enzyme	0.26
Native enzyme + 4 M GdnHCl	3.45
Native enzyme + 6 M GdnHCl	3.44
Ligand-linked enzyme	0.04
Ligand-linked enzyme + 4 M GdnHCl	0.23
Ligand-linked enzyme + 6 M GdnHCl	3.37

linked enzymes respectively. These values are similar to those reported for the *E. coli* enzyme (Kleanthous et al., 1991). As also observed for the *E. coli* enzyme, small changes occur in the ellipticity of the ligand-linked enzyme over the range of concentrations of GdnHCl from 2 M to 4 M, before the major change occurs. For the *E. coli* enzyme it was suggested that these initial small changes might reflect dissociation of the dimer before more extensive unfolding of each subunit (Kleanthous et al., 1991).

The fluorescence changes, which monitor the loss of tertiary structure in the environment of the tryptophan side chain in each subunit, also show that the ligand-linked enzyme is considerably more stable than the native enzyme (Figure 6). For the native enzyme, the changes in c.d. and fluorescence run broadly in parallel, with the loss of tertiary structure at a given concentration of GdnHCl being slightly greater than the loss of secondary structure. However, for the ligand-linked enzyme a rather different pattern is observed. In the region from 2 M to 4 M GdnHCl, a considerably greater loss of tertiary structure than of secondary structure is observed. This behaviour could indicate that one or more intermediates with some of the characteristics of 'molten globules' (Christensen and Pain, 1991) are involved in the unfolding process. However, there was no significant change in the enhancement (approx. 1.5-fold) of fluorescence of ANS in the presence of either native or ligand-linked enzyme over the range of GdnHCl concentrations from 0 to 6 M, suggesting that 'molten globule' states are not significant. An alternative explanation could be that dehydroquinase contains multiple domains which unfold independently; in this case changes in ellipticity and fluorescence would not necessarily be correlated. It has been suggested that the unfolding of *E. coli* type I dehydroquinase could also be interpreted in terms of such a multiple-domain model (Kleanthous et al., 1991).

Quenching of tryptophan fluorescence

The efficiency of quenching of protein fluorescence by agents such as succinimide can be taken as a measure of the degree of accessibility of the fluorophore (Eftink and Ghiron, 1984). The presence of the single tryptophan side chain in *S. typhi* dehydroquinase affords an excellent opportunity to monitor the environment of a defined locus (i.e. a moderately exposed side chain) in the enzyme. As shown by the magnitudes of the Stern-Volmer constants (Table 2), there is a dramatic difference between the degree of quenching in the native and ligand-linked enzymes, with the respective Stern–Volmer constants being 0.24 M^{-1} and 0.04 M^{-1} respectively. In the latter case the tryptophan side chain is much less accessible to the quencher, consistent with the tightening of the structure indicated by the GdnHCl-denaturation and the DSC data. When acrylamide is used as the quenching agent, the Stern–Volmer constant for the ligand-linked enzyme is only 3-fold lower than for the native enzyme (0.09 M^{-1} compared with 0.27 M^{-1}).

On addition of GdnHCl at concentrations which lead to unfolding of the enzymes, the Stern–Volmer constants for succinimide increase considerably and become of comparable magnitude for the native and ligand-linked enzyme (Table 2). These values are comparable with that for quenching of the fluorescence of the model compound *N*-acetyltryptophan amide by succinimide in the presence of 6 M GdnHCl (5.2 M^{-1}). The retention of considerable folded structure by the ligand-linked enzyme in the presence of 4 M GdnHCl is shown by the low value of the Stern–Volmer constant under these conditions (Table 2).

DSC

DSC experiments (Figure 7) on the native enzyme show a single, sharp, endothermic unfolding transition in the 55–56 °C temperature range. At relatively high concentrations this transition is accompanied by exothermic irreversible protein aggregation, which distorts the thermogram and makes detailed interpretation difficult. However, at low protein concentrations this aggregation is delayed sufficiently to allow deconvolution of the thermal unfolding in terms of standard two-state models. The calorimetric enthalpy of unfolding, obtained from integration of the excess heat capacity and expressed per mol of protein monomer, is about 370 $kJ \cdot mol^{-1}$, compared with a van't Hoff enthalpy, determined from the shape of the transition, of roughly twice this (704 $kJ \cdot mol^{-1}$). This factor of 2 is consistent with a dimeric co-operative unit, and indicates that the protein dimer remains

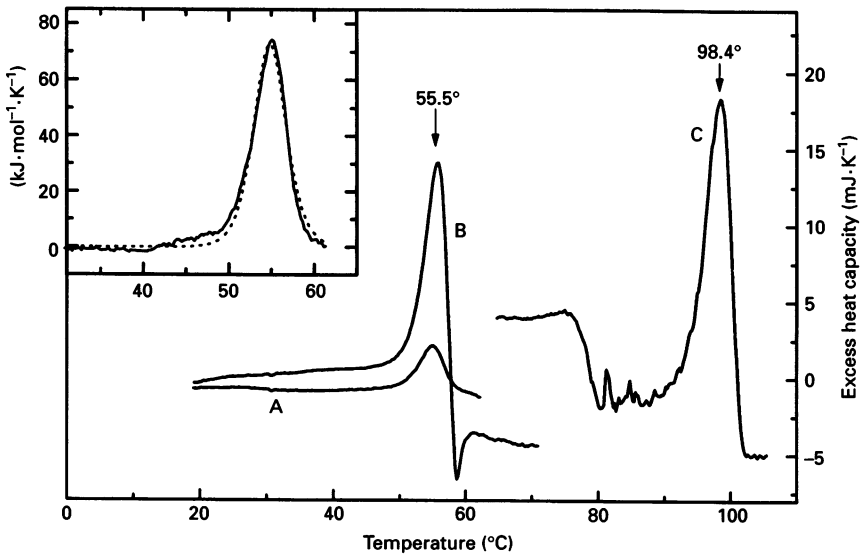

Figure 7 DSC thermograms of *S. typhi* dehydroquinase in 50 mM potassium phosphate, pH 7.0, after buffer baseline correction

Traces: A, native enzyme (0.87 mg/ml); B, native enzyme (2.9 mg/ml); C, ligand-linked enzyme (2 mg/ml); insert, concentration-normalized excess-heat-capacity data for the native enzyme at low concentration (0.87 mg/ml), normalized per mol of monomer, and the fit in terms of a single two-state transition. The broken line is the theoretical plot with T_m = 55 °C, calorimetric enthalpy (ΔH_{cal}) = 370 kJ·mol^{-1} (monomer) and van't Hoff enthalpy (ΔH_{VH}) = 704 kJ·mol^{-1}.

intact until thermally denatured. Just as with the *E. coli* dehydroquinase (Kleanthous et al., 1991), formation of the ligand-linked enzyme gives a remarkable enhancement in thermal stability of the enzyme, increasing T_m (apparent melting temperature) by over 40 °C (Figure 7). Preliminary analysis indicates that the data are consistent with a dimeric co-operative unit for the ligand-linked *S. typhi* enzyme, which apparently contrasts with earlier experiments on the *E. coli* enzyme that suggested that dimer dissociation precedes unfolding in this case (Kleanthous et al., 1991). However, this interpretation should be treated with caution, since these high-temperature DSC transitions may be distorted by the aggregation of the protein observed at all protein concentrations tried so far.

DISCUSSION

X-ray-crystallographic studies on the type I dehydroquinase from *S. typhi* are well underway (Boys et al., 1992), with the prospect of a detailed analysis of (i) the mechanism of this class of enzymes, which involves an unusual *cis*-elimination of water, and (ii) the structural basis of the extraordinary stability of the ligand-linked form of the enzyme. However, it is important to establish that the conclusions drawn from the *S. typhi* enzyme can be applied to other type I dehydroquinases, including the *E. coli* enzyme, which has been investigated in considerably greater detail. The results presented in this paper confirm that the kinetic and mechanistic properties of the enzymes from *S. typhi* and *E. coli* are very similar. The two enzymes have similar kinetic parameters and, on the basis of chemical-modification and pH–rate data, the active site in each case has been shown to contain a histidine and a lysine side chain. The c.d. spectra confirm that the secondary structures of the two enzymes are also very similar. Formation of the ligand-linked enzyme leads in both cases to a dramatic increase in stability towards chemical or thermal denaturation. As previously observed for the *E. coli* enzyme (Kleanthous et al., 1991), this stabilization involves little or no change in secondary structure and only a small change in tertiary structure, as revealed by near-u.v. c.d. or fluorescence. The unfolding of the ligand-linked *S. typhi* dehydroquinase by GdnHCl has been monitored by both far-u.v. c.d. and fluorescence measurements. The marked non-coincidence of the changes in these parameters suggests that the enzyme consists of at least two domains which unfold separately, as suggested for the *E. coli* enzyme (Kleanthous et al., 1991). Although the formation of the ligand-linked enzyme involves only minor changes in structure, it does lead, in the case of the *S. typhi* enzyme, to a remarkable decrease in the accessibility of the single tryptophan side chain to succinimide, as indicated by the 6-fold decline in the Stern–Volmer constant (Table 2). Since the degree of exposure to water, as reflected in the emission maximum, is effectively the same in native and ligand-linked enzymes, the decrease in the Stern–Volmer constant must imply that the ligand-linked enzyme has a much more rigid structure, so that the localized movements which allow succinimide to penetrate into the interior of the protein are greatly restricted. This conclusion is supported by the results with the smaller quencher, acrylamide, where the Stern–Volmer constant for the ligand-linked enzyme is only 3-fold lower than for the native enzyme.

The 'tightening' of protein structure by additional interactions such as salt bridges and hydrogen bonds has been proposed to be an important mechanism by which thermophilic enzymes can achieve thermal stability (Jaenicke, 1991; Varley and Pain, 1991). At temperatures approaching the optima for thermophilic enzymes, their flexibilities have increased to a level comparable with those of mesophilic enzymes at lower temperatures, resulting in similar values for catalytic constants. For the ligand-linked dehydroquinases from *E. coli* and *S. typhi* the tightening of structure is reflected in an increase of over 40 °C in the melting temperature and a 3-fold increase in the mid-point concentration of GdnHCl required to cause loss of secondary structure. Recently, we have obtained crystals of the ligand-linked dehydroquinase, which will allow us to compare the detailed structures of the native and ligand-linked forms. The data in this paper show that the two forms have very similar secondary and tertiary

structures. Comparisons of the detailed X-ray structures will allow the identification of the specific interactions which contribute to the enhanced stability of the ligand-linked enzyme. The type I dehydroquinase system provides an excellent opportunity to delineate, at the molecular level, a mechanism of achieving thermal stability in proteins.

We thank the Science and Engineering Research Council for financial support and a studentship to J.D.M., Dr. Colin Kleanthous for helpful discussions and Dr. D. M. Mousdale (Bioflux Ltd.) for performing the amino acid analysis. R.D. is a visiting scholar funded by the Government of Assam, India.

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Received 31 March 1993/24 May 1993; accepted 3 June 1993

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