Mutagenesis of Active Site Residues in Type I Dehydroquinase from Escherichia coli

STALLED CATALYSIS IN A HISTIDINE TO ALANINE MUTANT*

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Chemical modification experiments have previously implicated four amino acid residues in the mechanism of type I dehydroquinase from Escherichia coli. To further test their importance, these residues were mutated, and the resulting mutants were expressed, purified, and characterized. When the highly conserved, Schiff base-forming lysine residue was mutated (K170A) the resulting enzyme showed a ~10-fold reduction in catalytic activity, but was still able to bind both substrate and product, as shown by a novel fluorescence-based ligand-binding assay. This is consistent with Lys-170 playing a central role in catalysis and shows that, although forming a covalent bond with the substrate, it is not essential for ground state binding of substrate or product. Conversely, substituting leucine for the conserved, iodoacetate-reactive methionine residue (M205L) had little effect on \( k_{cat} \) or \( K_m \). Diethylpyrocarbonate experiments had previously implicated either His-143 or His-146 as the putative active site general base. Substituting alanine for each shows that H146A retains full catalytic activity while H143A shows a 10-fold loss of activity. As with the K170A mutant, H143A can bind ligand, and in addition to the predicted role of this residue as the proton-abstracting general base, our data suggest that it is also involved in the formation and breakdown of Schiff base intermediates. Isoelectric focusing, electrospray ionization mass spectrometry, and fluorescence spectroscopy show that the H143A mutant preferentially stabilizes the formation of the product Schiff base, and that this results in burst kinetics reminiscent of p-nitrophenyl acetate hydrolysis by chymotrypsin. The most striking illustration of this stabilization is the fact that the H143A mutant is isolated from overexpressing cells with a significant proportion of the enzyme monomers covalently bound to the product, 3-dehydroshikimate, via a Schiff base linkage. Our data suggest that the H143A mutant is able to slowly transform substrate to product but that the hydrolytic release of the product is stalled. The proposed dual role of His-143 in the mechanism of type I dehydroquinase may explain why the elimination reaction catalyzed by this enzyme proceeds with syn stereochemistry.

Dehydroquinase (DHQase; 3-dehydroquinase dehydratase) catalyses the dehydration of 3-dehydroquinic acid to 3-dehydroshikimic acid and is the third step in the central shikimate pathway of microorganisms, fungi, and plants (1). The end products of the pathway are the precursors of the aromatic amino acids, and so the enzymes involved in these biosynthetic reactions have been studied as potential sites for antimicrobial agents and herbicides (2). The focus of the present work is the mechanism of the dehydroquinase enzyme from Escherichia coli (EC 4.2.1.10). In particular, the roles of several amino acid residues have been investigated which chemical modification experiments have implicated as either being involved in the mechanism or as being in close proximity to the active site.

There are two distinct classes of DHQase enzymes, designated type I and type II, whose properties and mechanisms have been compared previously (3). Type II DHQases are thermolabile enzymes with a relatively small subunit molecular mass (~16 kDa) but which oligomerize to form dodecamers. They were originally identified as part of a catabolic pathway for utilization of quinic acid in fungi (4), but have since been found in the shikimate pathway of a number of prokaryotes (5-7). The stereochemistry of dehydration for the type II enzyme of Anaoycistis nidulans has been identified as anti (8), and it is thought that the elimination of water occurs through a concerted mechanism without the involvement of covalent intermediate (3, 9). The type I class, typified by the E. coli and Salmonella typhi enzymes, are dimers of 25-kDa monomer molecular mass and are not heat-stable (10, 11). The E. coli enzyme in particular has been studied extensively, and the current model for its mechanism is shown in Scheme 1. Chemical modification and peptide mapping studies have established that Lys-170 forms a Schiff base with the substrate and product; the latter intermediate can be trapped by reduction with sodium borohydride (12, 13). It has also been shown that reduction of this intermediate has profound effects on the stability of the resulting protein (14, 15). Lys-170 is conserved in all the known type I DHQase sequences (11, 13, 16, 17), which is consistent with its central role in the mechanism. Stereochemical experiments have shown that, unlike the reaction catalyzed by the type II class, the reaction is a syn elimination (9, 18). Since anti elimination is chemically the preferred course for the uncatalyzed reaction (18), it has been suggested that the formation of the Schiff base involves some distortion of the carbocyclic ring of dehydroquininate to render the pro-R proton more reactive (9).

The pH dependence of \( V_{max} \) for E. coli DHQase reveals the
presence of a single ionizing species with a $pK_a$ of 6.2 (10), consistent with the action of a general base in the mechanism (Scheme 1). The identity of this base has been inferred from diethyl pyrocarbonate experiments in which the modification of a single histidine residue led to inactivation of the enzyme, and the $pK_a$ of this modification corresponded to the $pK_a$ determined from the pH-$V_{max}$ profiles of the enzyme (19). Peptide mapping of the modified enzyme and sequencing of an isolated peptide established that the reactive histidine residue was either His-143 or His-146, and on the basis of sequence comparisons it was concluded that His-143 was the putative general base.

Iodoacetate acts as an affinity labeling reagent of E. coli DHQase, destroying activity by alkylating two unusually reactive methionine residues, Met-23 and Met-205 (20, 21). Of the two residues, only Met-205 is completely conserved in the type I family. Alkylation of both residues is required for complete inactivation of the enzyme, implying that these residues may not be crucial for enzyme activity but may lie close to the active site. Moreover, the carboxymethylation of Met-205 can be reversed by treatment with 2-mercaptoethanol but only when the enzyme is in the folded state (21). This was interpreted by Kleanthous and Coggins (21) as demonstrating the close proximity of a neutralizing positive charge near the carboxylate moiety of the carboxymethylated Met-205.

At present, there are no three-dimensional structures for either class of DHQase, but high resolution data should become available in the near future, since diffracting crystals of both a type I (22) and a type II (23) enzyme have been produced. In the absence of such data the roles of all the amino acids thus far identified by chemical modification need clarification. Toward this end we have mutated residues His-143, His-146, and Lys-170 to alanine and Met-205 to leucine and characterized the resulting mutant proteins to elucidate further the roles of the wild type residues in the mechanism of type I DHQase. The mutations addressed several as yet unanswered questions concerning the roles of these residues. The histidine mutations were made for two reasons, first to determine unambiguously which of the two was the likely DEPC-reactive residue and second to assess the properties of the essential histidine to alanine mutation in terms of the known mechanism. Met-205 alone was mutated since it is the only one of the two reactive methionine residues which is conserved in the type I family and Met-23 has already been mutated and shown to have no effect on enzyme activity.2 Alanine was substituted for the Schiff base-forming lysine to assess the relative importance of this residue in catalysis and ground state binding of ligand.

**Materials and Methods**

**Chemicals—** Ammonium 3-dehydroquinate was prepared by the method of Grewe and Haendler (24). Ammonium 3-dehydroshikimate was prepared by treatment of 3-dehydroquinate with DHQase (this produces a 15:1 dehydroshikimate:dehydroquinate mixture). Hydroxylamine hydrochloride was from Sigma. DE52 ion exchange resin was from Whatman. Other chemicals were of reagent grade or better and used without further purification.

**Genetic Materials—** Plasmid pKD203 (which contains the 1.8-kilo base pair genomic fragment from pKD201 (25) ligated into pKK223-3) was a kind gift of Dr. K. Duncan. The expression vector pKK223-3 was from Pharmacia Biotech Inc. E. coli strain AB2848 (tax-356, supE42, λ-, araD352) (26) was obtained from Dr. B. Bachmann at the E. coli Genetic Stock Center. Mutagenic, PCR, and sequencing primer oligonucleotides were synthesized on a New Brunswick Cyclone synthesizer.

Overexpression of 3-Dehydroquinase—The araD gene encoding the type I DHQase from E. coli was isolated, free of extraneous chromosomal DNA, from plasmid pKD203 by PCR using primers complementary to the ends of the open reading frame. The primers were extended with a sequence designed to place an EcoRI restriction site at the 5'-end of the gene and a HindIII site at the 3'-end. The blunt-ended PCR fragments were ligated into a pUC18-derived cloning vector which was propagated in E. coli. Clones containing inserts were selected by the loss of β-galactosidase activity. The araD fragment was then isolated by restriction digestion with EcoRI and HindIII and ligated into the expression vector pKK223-3. The resulting construct was introduced into E. coli JM105 by transformation. Plasmid DNA from clones containing an insert was checked for appropriate size and restriction sites. Following DNA sequencing of the araD gene using an A.L.F automated sequencer (Pharmacia), suitable clones were induced with isopropyl thiogalactoside and one (pAL2-2) producing a band of suitable size on SDS-PAGE was selected. This plasmid was then introduced into the araD point mutant strain, E. coli AB2848. For protein preparation, this clone was grown on LB medium in the presence of 200 μg/ml ampicillin to an OD$_{600}$ of 0.7–0.8, whereupon expression was induced with 1 mM isopropyl thiogalactoside. The cells were grown for a further 6 h and harvested by centrifugation at 5000 × g for 10 min.

In Vivo Complementation Tests—Strain AB2848 was inoculated onto M9 minimal agar plates with and without aromatic amino acids (40 μg/ml phenylalanine, tyrosine, and tryptophan). Bacteria harboring the plasmids with enzymically active araD genes (wild type, M205L, and H146A) grew on both plates. Those with no active araD gene (pK223-3 vector and K170A) grew only when supplemented with aromatic amino acids. The H143A mutant gave rise to an intermediate phenotype in that the bacteria grew well on supplemented medium but very slowly without aromatic amino acids.

**Purification of Dehydroquinase—** Purifications of overexpressed type I wild type and mutant DHQase enzymes were carried out by a procedure modified from that first described by Chaudhuri et al. (10). Cells grown in LB medium were resuspended in buffer (50 mM Tris-HCl, 5 mM MgCl$_2$, 1 mM EDTA, 1.2 mM phenylmethylsulfonyl fluoride, pH 8.0; 40 mM/iter of culture harvested) and treated with lysozyme (0.1 mg/ml) for 30 min at 37 °C. The cells were then disrupted by three freeze-thaw cycles. The DNA and RNA in the viscous mixture were hydrolyzed by incubation with DNase I and RNase A (0.05 mg/ml) for 30 min at 37 °C. After centrifugation at 21,000 × g for 30 min at 4 °C, the supernatant was taken to 75% saturation with ammonium sulfate. The precipitate was recovered by centrifugation (21,000 × g, 30 min), redissolved in low salt buffer (50 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.5) and dialyzed extensively against the same buffer. The protein solution was then applied to a 50-m column of DE52 anion exchange resin previously equilibrated in the same buffer. Dehydroquinase was eluted with a salt gradient (50–250 mM KCl: elution at 100–900 mM salt). Fractions were assessed by SDS-PAGE and enzyme activity and were usually more than 85% pure at this stage. Yields were up to

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2 E. Borthwell and J. R. Coggins, unpublished observations.
100 mg/liter of culture. For further purification, these fractions were dialyzed against potassium phosphate buffer, pH 7.0, concentrated to ~4 mg/ml in an Amicon concentrator and then applied to a semi-preparative Superdex-75 gel filtration column (360 ml; 26 mm × 600 mm) equilibrated in the same buffer. Enzyme prepared this way was typically >95% pure by SDS-PAGE. A further treatment of the H143A protein involved dialyzing the purified enzyme against 50 mM phosphate buffer, pH 7.0, containing 50 mM hydroxylamine which removed Schiff base-linked product molecules (see “Isoelectric Focusing” under “Results”).

Protein Determinations—Protein concentration determinations were carried out by the method of Bradford (27), using a kit from Bio-Rad, with bovine serum albumin as a standard. Protein concentrations were determined by spectrophotometry using the extinction coefficient ε 280 = 13,600 at 280 nm (19).

Mutagenesis—A modified M13 method was used for the generation of site-directed mutants (28). Mutants were selected by single track sequencing and confirmed by complete sequencing of the aroD gene. The mutant genes were ligated into pKK223-3 and overexpressed as described above. The resulting proteins were all similar in size to wild type enzyme by SDS-PAGE. Mutations were confirmed by accurate mass measurement using ESI-MS (see below).

Enzyme Assay—Dehydroquinase activity was assayed by monitoring the appearance of product by spectrophotometry at 234 nm as described previously (10). For determination of kinetic parameters, seven concentrations of dehydroquinate spanning the range 0.1 to 10 mM were employed, and the resulting data were fitted to a single exponential decay. All experiments were done in 20 mM potassium phosphate buffer, pH 7.0, containing 50 mM hydroxylamine and 50 μM hydroxylamine which removed Schiff base-linked product molecules (see “Materials and Methods”). Using this expression construct both wild type and mutant DHQases were transformed into the aroD point mutant AB2848, a strain that was first described by Pittard and Wallace (26). AB2848 was chosen since a suitable E. coli aroD deletion mutant was unavailable. AB2848 was auxotropic for aromatic amino acids and so was used as an initial assay for functional DHQase. Enzymatically active mutants (H146A and M205L) were able to complement the aroD phenotype of E. coli AB2848 when grown on minimal medium in the absence of aromatic amino acids but the inactive K170A mutant did not; in the case of the H143A mutant, the growth on minimal medium was much slower. Wild type and mutant proteins were purified from AB2848 grown on LB medium as described under “Materials and Methods.” The mutations had relatively little effect on the physical properties of the proteins; all were overexpressed well without toxic effects or any observed degradation. They all behaved similarly on SDS-PAGE and on the ion exchange columns used for purification, and in preliminary experiments they were denatured at similar concentrations of guanidine hydrochloride to those reported previously for the wild type enzyme (3, 14). It is therefore unlikely that the specific effects seen are due to destabilization of the protein structure caused by any of the mutations.

Isoelectric Focusing—The purity of each of the mutant proteins was checked by SDS-PAGE and shown to be >95% pure (data not shown). As a matter of routine, the mutants were also analyzed by isoelectric focusing gel electrophoresis to determine whether any charge variants were present in the native protein preparations. Wild type DHQase showed a single band with a pI of 4.9 (in agreement with previous work) (30), and similar isoelectric points were determined for the K170A and M205S mutants (Fig. 1A, lanes 1, 3, and 4, respectively).

The H143A mutant showed a surprisingly complex pattern on IEF gels (Fig. 1A, lane 2); as prepared, it consists of three distinctly species ranging from pl 4.6 to 4.9. This pattern is reminiscent of that seen when the Schiff base intermediate in wild type enzyme is first reduced with sodium borohydride (to yield a modified dimer of pl 4.6) and mixed with unmodified enzyme under denaturing conditions, and the protein mixture is allowed to refold (30). In addition to the recovery of modified and unmodified dimers this treatment also results in the formation of a hybrid dimer (pl 4.75) in which one subunit carries a covalently bound product molecule while the other remains unmodified. Such a hybrid mixture was run as a control in Fig. 1A (lane 5). A very similar pattern is observed for the H143A mutant as purified (Fig. 1A, lane 2). However, in contrast to the hybrid mixture derived from wild type enzyme which is fixed, the extent of modification in the H143A mutant can be altered in several ways. 1) Unfolding of the protein in 6 M guanidine hydrochloride, followed by dialysis to remove the denaturant, completely removes the modification to yield a protein with the same pl as wild type dimer (Fig. 1B, lane 2). It is known that refolding of the wild type, dimeric enzyme occurs under these conditions with 100% recovery of folded protein (30). 2) Dialysis against pH 7.0 buffer containing 50 M hydroxylamine also


course experiments, and K_m determinations were performed using Enzfitter (31) or Ultrafit (32) software.

RESULTS

Overexpression and Purification of Dehydroquinases from AB2848—pkD203 contains the aroD gene flanked by approximately 1 kilobase pair of extrachromosomal DNA in the expression vector pK223-3. The level of expression from this construct is ~10 mg/liter. However, we found that removing the flanking chromosomal DNA by PCR and cloning back into pK223-3 increased the level of expression to ~100 mg/liter (see “Materials and Methods”). Using this expression construct both wild type and mutant DHQases were transformed into the aroD point mutant AB2848, a strain that was first described by Pittard and Wallace (26). AB2848 was chosen since a suitable E. coli aroD deletion mutant was unavailable. AB2848 is auxotropic for aromatic amino acids and so was used as an initial assay for functional DHQase. Enzymatically active mutants (H146A and M205L) were able to complement the aroD phenotype of E. coli AB2848 when grown on minimal medium in the absence of aromatic amino acids but the inactive K170A mutant did not; in the case of the H143A mutant, the growth on minimal medium was much slower. Wild type and mutant proteins were purified from AB2848 grown on LB medium as described under “Materials and Methods.” The mutations had relatively little effect on the physical properties of the proteins; all were overexpressed well without toxic effects or any observed degradation. They all behaved similarly on SDS-PAGE and on the ion exchange columns used for purification, and in preliminary experiments they were denatured at similar concentrations of guanidine hydrochloride to those reported previously for the wild type enzyme (3, 14). It is therefore unlikely that the specific effects seen are due to destabilization of the protein structure caused by any of the mutations.

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removes the modification and again results in a protein with the same pI as wild type (Fig. 1B, lane 3). 3) Incubation with substrate under equilibrium conditions increases the extent of modification such that only modified dimer is now apparent (Fig. 1B, lane 4). Wild type enzyme under similar conditions (in which the Schiff base is not reduced) simply behaves as unmodified enzyme (Fig. 1B, lane 5). H143A mutant which had been cleared of endogenously bound ligand by hydroxylamine dialysis or guanidine hydrochloride denaturation can also be remodified with substrate, indicating that the removal of the modification is not an irreversible consequence of these treatments, and completely modified protein can be returned to the unmodified state proving that the denaturants do not selectively destroy modified protein (data not shown). In all subsequent experiments on this mutant the heterogeneity was removed by prior dialysis against buffers containing hydroxylamine.

The H146A mutant was also analyzed by isoelectric focusing (Fig. 1C, lane 1). Unlike the H143A mutant, a single isoelectric variant was observed. However, this mutant is clearly more acidic than H143A (pI 4.6), a property that is distinctly different from all the other mutations generated in this study; these all behaved like the wild type enzyme on IEF. In addition, borohydride reduction of ligand was required to trap the product Schiff base (again, unlike H143A) which further reduced the pI of the protein to 4.3 (Fig. 1C, lane 2). It is interesting to note that of the three mutations, H143A, H146A, and K170A, which could potentially increase the acidity of DHQase by removing a positive charge, only the H146A substitution produced this effect. Although circumstantial, this suggests that His-143 and Lys-170 do not carry positive charges at the isoelectric point of the native enzyme.

Steady State Kinetic Properties of the Enzymes—Wild type DHQase purified from AB2848 had Km and kcat values very close to published values (3) (Table I). For the K170A mutant, rates of product formation at 100 μM dehydroquinine (a concentration which is saturating for the wild type enzyme) were typically 10^6-fold lower than that of wild type. It was difficult to obtain accurate and reliable kinetic data on this mutant for several reasons. First, the amount of protein needed to obtain a steady state rate interfered significantly with the UV spectrophotometric assay (at 234 nm) normally employed to assay DHQase; second, the activity varied between preparations; and third, the activity could be abolished by reduction with sodium borohydride in the presence of substrate. This latter observation implies that these very low rates may be due to contamination with very small quantities of wild type enzyme. Given these limitations it is possible to state only that the K170A mutant is at least 10^6-fold less active than wild type enzyme. The source of these variable contaminations is unclear. The
most likely explanation is the araD point mutant strain which was used for the expression of the mutants; Schimmel (33) has highlighted several potential caveats on using such strains to express mutant genes, including the possibility of structural complementation of inactive protein partners, recombination, and translational misreading. However, regardless of the complication of the genetic background in which this and the other mutants were expressed, our data clearly indicate that substituting the active site lysine for alanine severely debilitates the catalytic activity of the enzyme.

The M205L mutant had a slightly increased $K_m$ and decreased $k_{cat}$ compared to wild type enzyme (Table I); $k_{cat}/K_m$ shows a decrease of ~3.5-fold. These changes are small in comparison with those seen for mutations of the other supposed active site residues and indicate that while Met-205 may be close to the active site, it does not play any significant role in the mechanism of the enzyme.

The H146A mutant had $K_m$ and $k_{cat}$ values very similar to wild type enzyme (Table I). Replacement of this amino acid with alanine clearly has little effect on the enzyme, ruling it out as the site of diethyl pyrocarbonate modification and as a mechanistically important residue.

Kinetic analysis of the H143A mutant required that it was "cleared" of endogenously bound ligand. This was accomplished (as described above and under "Materials and Methods") by a 24-h dialysis against hydroxylamine-containing buffer followed by removal of the hydroxylamine by a further dialysis step. As with the lysine mutant, homogenous H143A was practically inactive under normal assay conditions. However, unlike the K170A mutant, when large quantities of enzyme (50–200 $\mu$g/ml; 2–8 $\mu$M) were used in the assay, an initial burst of absorption at 234 nm was observed, which corresponded to the formation of 0.3–0.5 equivalents of product (based on the extinction coefficient of free 3-dehydroshikimate), followed by a very slow steady state increase (Fig. 2). The initial phases of these traces could be fitted to a single exponential, giving rates of $2.6 \times 10^{-3}$ s$^{-1}$ in each case. The steady state rates changed with substrate concentration in a hyperbolic fashion and, when they were fitted to the Michaelis-Menten equation, gave a $K_m$ similar to the wild type enzyme, whereas $k_{cat}$ was reduced by 5–6 orders of magnitude relative to wild type (Table I). It is possible that some of the residual activity observed in these preparations could be due to contamination with wild type enzyme. However, it seems very unlikely that this could be responsible for the slow burst at 234 nm that is observed with this mutant (wild type contamination would simply result in instant steady state rates). Furthermore, both mass spectrometry and fluorescence spectroscopy (see below) suggest that this slow burst represents accumulation of the product 3-dehydroshikimate at the active site of the H143A mutant.

Mass Spectrometry—Electrospray mass spectrometry has been used in previous studies both to evaluate the integrity of expressed DHQase and to analyze Schiff base formation in wild type enzyme (34). ESI-MS was also used in this study to address these points. Wild type DHQase purified from AB2848 yielded a single peak with a molecular mass of 27,467.2 ± 0.5 Da, consistent with the calculated value of 27,466.8 Da. On treatment with substrate and sodium borohydride, the mass increased by 156 units, corresponding to irreversible reduction of the product, 3-dehydroshikimate (data not shown), which is consistent with previous studies (34).

The masses for all mutants constructed in this study were analyzed by ESI-MS. The masses for H146A (observed 27,400.8 ± 0.2, calculated 27,400.7 Da), M205L (observed 27,448.7 ± 0.8, calculated 27,448.6 Da) and K170A (observed 27,409.32 ± 0.6, calculated 27,409.5 Da) all showed single peaks which corresponded to their predicted masses. In addition, K170A was incapable of forming a borohydride-susceptible Schiff base in the presence of substrate or product (data not shown). The purified H143A mutant does not show a single peak but two, one at 27,400.7 ± 0.7 Da, which corresponds to the calculated mass of the protein (27,400.6 Da), and the other at 27,554.6 ± 2.6 Da (Fig. 3A). The mass difference between these two peaks is 154 Da, which corresponds to the mass of the Schiff base of the product, 3-dehydroshikimate. Hydroxylamine-treated or guanidine hydrochloride-denatured enzyme (Fig. 3B) shows only one peak, at 27,401.2 ± 0.3 Da. When the H143A mutant which had been cleared of endogenously bound ligand was treated with substrate, a peak at 27,554.5 ± 0.7 Da reappeared and increased while that of the unmodified peak diminished (Fig. 3C).

The resolution of the ESI-MS data collected on all the DHQase samples (~2 Da) was sufficient to distinguish between product- and substrate-bound enzyme (which differ in mass by 18 Da). Nevertheless, we further analyzed the mass of the product-linked form of the H143A mutant using the maximum entropy software supplied with the mass spectrometer. Here again, only the product form was identified (data not shown).

We found that the solvent used for these mass spectrometric measurements (1:1:0.001 acetonitrile:water:formic acid) resulted in the slow removal of the modifying group that produces the peak at 27,555 Da, and so experiments were conducted in such a way that samples were added to the mobile phase of the mass spectrometer immediately before injection and using a high flow rate. Using this approach, it was possible to monitor the time-dependent accumulation of product on the H143A mutant following mixing with substrate (Fig. 4). These data could be fitted to a single exponential curve with a rate constant of $2.2 \times 10^{-3}$ s$^{-1}$. Mixing substrate with wild type enzyme also shows the appearance of a Schiff base-bound subunit (in agreement with the work of Shneier et al. (34)). This intermediate did not, however, accumulate with time but remained at a constant level (~10% of the unmodified peak).

Spectrofluorimetry—There have as yet been no reports in the
literature of spectroscopic methods for the detection of ligand binding to DHQases. Nevertheless, it was important in the characterization of the mutants generated in this study to be able to distinguish effects on catalysis from effects on ligand binding. This is especially true of the mutants with very low catalytic activities such as H143A and K170A. It had previously been observed that the fluorescence of type I DHQase is decreased following their reversible attachment of the product, 3-dehydroshikimate, by borohydride reduction (30). In view of this, the effect of ligand binding on the fluorescence of both wild type and the various mutants was tested under equilibrium conditions, without the addition of borohydride. In these experiments, fluorescence was excited at 295 nm which is selective for the single tryptophan of E. coli DHQase.

In most cases, it was possible to study only the effect of an equilibrium mixture of substrate and product ($K_{eq} = 15$; see "Materials and Methods" for further details) on the fluorescence properties of wild type and mutant enzymes, and the values resulting from this analysis are shown in Table I. Addition of such a mixture to wild type enzyme caused a decrease in fluorescence intensity but no significant change in the wavelength of maximum emission (325 nm), implying that there is no major change in the environment of the tryptophan residue (Fig. 5A). A plot of the change in intensity versus ligand concentration had the shape of a simple ligand binding curve from which a $K_d$ was derived (Table I).
For the K170A mutant the effects of substrate alone and the ligand mixture on protein fluorescence could be distinguished because substrate and product are not interconverted during the course of the experiment (Fig. 5B and Table I). It is notable from these data that the product is less strongly bound than the substrate in this mutant. Although a residue which makes a covalent bond to the substrate has been removed in the K170A mutant, the binding affinity for the equilibrium ligand mixture is only marginally affected.

The H143A mutant again shows features distinct from the other DHQase mutants. The substrate and the equilibrium ligand mixture can bind to this enzyme and cause a fluorescence quench, but this process was found to be time-dependent, and in both cases the decay could be fitted satisfactorily to a single exponential (see Fig. 6, where the curve for substrate is shown). At saturating concentrations of substrate (such as is shown in Fig. 6, see below) the rate of decay was $2.5 \times 10^{-3}$ s$^{-1}$. The rate constant determined for the substrate/product mixture was similar (data not shown). The overall magnitude of the ligand-induced fluorescence quench for H143A was equivalent to that of wild type enzyme and was found to be concentration-dependent. However, experiments conducted under equilibrium conditions (with substrate alone and with the ligand mixture) indicated that the dissociation constant was very low, and attempts to determine it required significantly lower protein concentrations than those used for the other proteins in this study, at which point spectrometer noise became a problem. It was therefore possible to say only that the $K_d$ for substrate alone (and the substrate/product equilibrium mixture) for this mutant lies below 1 $\mu$M. The fact that the fluorescence quench saturates at a given concentration of ligand indicates that it is likely to be the result of an event following the rapid binding of ligand.

**DISCUSSION**

The Mechanism of Dehydroquinase—Scheme 1 depicts a plausible mechanism for type I DHQases. A notable feature of the mechanism is its symmetry around the central elimination step. The equilibrium point for the reaction is reached at about 15:1 dehydroshikimate:dehydroquininate, and if supplied with pure dehydroshikimate the enzyme will catalyze the reverse reaction, when the $K_m$ for DHS is found to be 65 $\mu$M and $k_{cat} \sim 50$ s$^{-1}$ (35). We note that the apparent $K_a$ for the wild type enzyme (60 $\mu$M; Table I) for the DHQ/DHS mixture determined in this study is close to the estimated $K_m$ value for product. Experiments with substrate and product analogues have shown that the active site imposes relatively strict require-
Residues His-146 and Met-205 Are Not Catalytically Important—Replacement of Met-205 with leucine, a residue of similar size but lacking the sulfur atom, resulted in only a small decrease in activity (Table I), and this would be consistent with some form of conformational change at the active site of the enzyme. However, whether this alteration in structure represents the postulated conformational change resulting in distortion of the substrate carbocyclic ring or is simply a result of the close proximity of the substrate to the active site of DHQase, will be clear only when crystal structures of bound and free DHQase become available.

Removal of His-146 Abolishes Activity but Not Ligand Binding—Two active site residues are explicitly identified in Scheme 1; Lys-170 which forms the Schiff base and a general base B which catalyzes the elimination step, thought to be His-143 (19). By analogy with acetoacetate decarboxylase (37), formation of the Schiff base would be expected to increase the acidity of the C2 proton and, given the above observations, specifically the pro-R proton. The general base is then able to protonate the leaving hydroxyl group. However, the type II dehydroquinases perform the same transformation (but with opposite stereochemistry) apparently without a covalent intermediate, and so it is plausible that the imine linkage could act simply as a "tether" to hold the substrate in the active site.

The mutation of Lys-170 to alanine almost totally abolishes activity. This makes it clear that formation of the Schiff base is indispensable for catalysis in this enzyme; if it were simply acting as a tether for the substrate, one might expect some residual activity at high substrate concentrations, but this is not seen. The ligand binding studies, however, show that substantial binding interactions remain; the $K_d$ for substrate/product equilibrium mixture is only about 3-fold higher than the $K_d$ for wild type enzyme, and a low $K_d$ could be measured for substrate (Table I). This implies that the lysine must exert most of its influence on the catalytic steps of the enzyme, rather than the binding of substrate or product. It might be expected that the covalent bond between substrate and enzyme would make a significant contribution to ground state binding, yet it is clear that this is not the case; Lys-170 plays a predominantly catalytic role in the mechanism.

Removal of His-143 "Stalls" Catalysis—In the mechanism of Scheme 1, His-143 is postulated to act as a general base. Its removal might be expected to have several effects. If it were acting solely as a proton acceptor, loss of this function would abolish enzyme activity but still permit binding and release of substrate and product (i.e. Schiff base formation and hydrolysis), because elimination might be expected to become the ratetermining step. (It should be noted, however, that the rate-limiting step for wild type DHQase is still unknown). A further consequence of this might be that in the presence of substrate, the Schiff base-bound substrate would accumulate, whereas the product form of this covalent intermediate would accumulate if product were present. Therefore, on reduction with sodium borohydride the different Schiff base intermediates would be expected to appear in the ESI-MS experiments. In fact, whether substrate or product is added to the H143A mutant, only the product adduct can be detected (Fig. 3).

One of the major effects of substituting alanine for histidine at position 143, in the forward direction, is to dramatically slow down the rate at which the enzyme can form product at the active site. The ESI-MS data indicate that, in the presence of substrate, the product Schiff base accumulates at the active site with a rate constant of $2.2 \times 10^{-3} \text{ s}^{-1}$ (Fig. 4). A similar rate constant ($2.6 \times 10^{-3} \text{ s}^{-1}$) is obtained from the pre-steady state phase of the activity assays for H143A (Fig. 2), suggesting that this too represents the accumulation of the product at the active site. The amplitude of this burst phase corresponds to $<0.5$ equivalents of free product for each active site, but this may not represent the true extent of binding, as the extinction coefficient of the product bound to the enzyme is unknown. The slow accumulation of the product Schiff base at the active site is coincident with a time-dependent fluorescence quench (Fig. 6) that has a first order rate constant $(2.5 \times 10^{-3} \text{ s}^{-1})$, almost identical to the values obtained by ESI-MS and the activity assays.

All of the above observations would be consistent with the postulated role of His-143 being the proton-abstacting general base. However, the H143A mutant has another property which suggests that in fact it may also be involved in the breakdown (and possibly formation) of the Schiff base intermediates. The hydrolytic release of the product Schiff base from the active site of the mutant is dramatically affected, even more so than the rate of formation of this intermediate. As discussed above, the rate of product accumulation is $-2.5 \times 10^{-3} \text{ s}^{-1}$, and this is approximately 60,000-fold slower than the turnover number for...
the wild type enzyme. However, the $k_{cat}$ for the alanine mutant is at least 2 orders of magnitude slower than this (Table 1), indicating that $k_{cat}$ is governed by the hydrolysis of the product Schiff base in H143A. The most striking illustration of this product stabilization effect is the fact that the enzyme, as purified, is substantially modified by the product Schiff base, as shown by the IEF and ESI-MS data (Figs. 1 and 3). A further point to make is that the presence of His-143 at the active site of DHQase appears to destabilize ligand binding since substituting this residue for alanine increases the affinity for both substrate and product.

These phenomena, accumulation of product followed by its very slow release, are strongly reminiscent of the classic experiments of Hartley and Kilby (38) on the burst kinetics of chymotrypsin when assayed with ester substrates. The rate-limiting step in the hydrolysis of esters by chymotrypsin is deacylation of the acyl-enzyme intermediate, and this is readily observed with poor substrates such as p-nitrophenyl acetate (38). The burst kinetics of the H143A DHQase mutant therefore consistent with the hydrolytic release of the product, 3-dehydroshikimate, being the rate-limiting step for this enzyme in the forward direction.

So what is the role of His-143 in the mechanism of type I DHQase? Our data are consistent with this residue having a wide ranging influence in the mechanism of the enzyme, involving the central steps of base-catalyzed elimination (Scheme 1) as well as the formation and breakdown of the Schiff base intermediates, depicted in Scheme 2. In this latter role, His-143 is proposed to act as a general acid in the formation of the imine intermediate and then a general base in its hydrolysis. An appealing aspect of this mechanism is that, following its participation in either formation or hydrolysis of the covalent intermediates, it remains appropriately placed and in appropriate ionization state, for its subsequent role in the central steps of the mechanism. In addition, a single base at the active site, involved in both C2 proton abstraction and Schiff base formation, may also explain why the type I DHQase reaction proceeds with syn stereochirality.

The idea of a dual function for His-143 in the type I DHQase mechanism has precedent in the literature of aspartate aminotransferase. The elegant studies of the K258A mutant of aspartate aminotransferase by Toney and Kirsch (39, 40) showed that exogenous amines could perform the 1,3-prototropic shift catalyzed by the active site lysine in the wild type enzyme, which is formally analogous to the elimination step of type I DHQase. Furthermore, exogenous amines also catalyzed hydrolysis of the Schiff base formed from aspartate and the pyridoxal cofactor at the active site of the mutant enzyme. Preliminary attempts to demonstrate this external amine catalysis with H143A DHQase have been unsuccessful (data not shown). If such effects are operating they are below the background rates we are currently able to measure.

In conclusion, using site-directed mutagenesis we have investigated the properties of mutant enzymes in order to probe the mechanism of type I DHQase from E. coli. Two of the mutations (H146A and M205L) show unambiguously that these residues play no role either in catalysis or ligand binding. Substitution of the active site Schiff base-forming lysine residue for alanine renders the enzyme virtually inactive but still capable of binding both substrate and product. The role of the putative active site general base His-143 would appear to be more complex than previously thought since substituting it for alanine increases the enzyme’s affinity for both substrate and product, drastically slows down the rate of product accumulation in the forward direction, and stalls the hydrolytic release of product from the active site of the enzyme.

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