Localization of the Active Site of Type II Dehydroquinases

IDENTIFICATION OF A COMMON ARGinine-CONTAINING MOTIF IN THE TWO CLASSES OF DEHYDROQUINASES

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A novel method based on electrospray mass spectrometry (Krell, T., Pitt, A. R., and Coggins, J. R. (1995) FEBS Lett. 360, 93–96) has been used to localize active site residues in the type I and type II dehydroquinases. Both enzymes have essential hyper-reactive arginine residues, and the type II enzymes have an essential tyrosine residue. The essential hyper-reactive Arg-23 of the Streptomyces coelicolor type II enzyme has been replaced by lysine, glutamine, and alanine residues. The mutant enzymes were purified and shown by CD spectroscopy to be structurally similar to the wild-type enzyme. All three mutant enzymes were much less active, for example the $k_{cat}$ of the R23A mutant was 30,000-fold reduced. The mutants all had reduced $K_m$ values, indicating stronger substrate binding, which was confirmed by isothermal titration calorimetry experiments. A role for Arg-23 in the stabilization of a carbanion intermediate is proposed. Comparison of the amino acid sequence around the hyper-reactive arginine residues of the two classes of enzymes indicates that there is a conserved structural motif that might reflect a common substrate binding fold at the active center of these two classes of enzyme.

It is generally believed that enzymes have evolved to catalyze reactions by optimal mechanisms. With the exception of the four classes of proteinases (1) and the two classes of aldolases (2), examples of mechanistically different pairs of enzymes that catalyze the same reaction are very rare. This unusual situation is found with the two classes of dehydroquinase that catalyze the dehydration of 3-dehydroquinate to form 3-dehydroshikimate (3). The reaction is common to two metabolic pathways: the biosynthetic shikimate pathway that is used for the synthesis of aromatic compounds in plants and micro-organisms (4) and the catabolic quinate pathway that enables fungi and some other micro-organisms to use quinate as a carbon and energy source (5). The type I dehydroquinases (DHQase) catalyze a cis elimination and are only involved in the biosynthesis of shikimate (6), whereas the type II enzymes, which catalyze a trans elimination (1), have been found to have either a biosynthetic (7) or a catabolic role (8) and in at least one species a dual role (9).

Besides being mechanistically distinct, the two classes of DHQase have very different biophysical properties and are apparently unrelated at the level of primary structure (10, 11). The type I enzymes are dimers with a molecular mass of about 46,000 Da; they are heat-labile and use a mechanism that involves the formation of a Schiff-base intermediate followed by the abstraction of a proton by a general base (12–15). In the case of the type I Escherichia coli enzyme the lysine residue has been located (Lys-170) by trapping the Schiff-base intermediate by borohydride reduction (12, 13), and this residue is conserved in all type I sequences (12) as is His-143 (15, 16) which is the general base. Both Met-23 and Met-205 (17) have also been identified as active site residues although their role in substrate binding or catalysis has not been established.

In contrast the type II enzymes are heat-stable dodecamers with a subunit molecular mass of about 16,000 Da (11); they catalyze a trans elimination of the elements of water (3); there are no conserved lysine residues, and they are resistant to inhibition by borohydride treatment.2 Clearly the type II enzymes do not use the Schiff-base mechanism, and it has been suggested that the two classes of DHQases are the result of convergent or parallel evolution (18).

Both the type I and type II enzymes have been crystallized (19, 20), and to aid structural analysis we have been using group-specific chemical modification to localize the active sites. Recently we have described a new method, based on electrospray mass spectrometry, for monitoring the modification of proteins by the arginine-directed reagent phenylglyoxal (PGO) (21). This method allows the direct measurement of the amounts of individually modified enzyme species. PGO rapidly inactivates the type II DHQases (21). In the early stages of inactivation only one modified species could be detected that correlated directly with the activity loss. The single site of modification was identified by HPLC-electrospray mass spectrometry-based peptide mapping to be the hyper-reactive residue Arg-23. Here we report the use of this methodology to identify further active site residues in the DHQases. Inactivation with tetratinromethane (TNM) has identified an essential tyrosine in the type II enzymes, and inactivation with PGO has identified a hyper-reactive arginine in the type I enzymes. The essential role of the active site arginine in the type II enzymes has been confirmed by site-directed mutagenesis and a number of mutant enzymes characterized kinetically, by circular dichroism (CD) and by isothermal titration calorimetry (ITC).
EXPERIMENTAL PROCEDURES

Purification and Assay of Type I and Type II DHQases—The type II DHQases from Streptomyces coelicolor (22) and Aspergillus nidulans (23) were overexpressed in E. coli and purified as described previously (24).3 Protein concentrations were determined spectrophotometrically at 280 nm using extinction coefficients calculated from the amino acid compositions (26).

The E. coli type I DHQase was purified from an overproducing strain according to the procedure of Chaudhuri et al. (6). Enzyme activity was determined by monitoring the formation of 3-dehydroshikimate at 234 nm (ε = 12 × 10^5 M^-1 cm^-1) at 25 °C. The assay mixture for the type I enzyme contained 100 μM ammonium dehydroquininate as substrate in 100 mM potassium phosphate (pH 7.0); for the type II enzyme of A. nidulans the assay mixture contained 1 mM ammonium dehydroquininate in 50 mM Tris acetate (pH 7.0). The assay for the type II enzyme of S. coelicolor was carried out in 50 mM Tris acetate (pH 8.0) containing 5 mM substrate.

Inactivation with Phenylglyoxal (PGO)—Enzyme inactivation reactions were carried out at 25 °C in a volume of 2 ml with continuous stirring. Samples of type I and type II DHQase (3 ml) in 100 mM sodium bicarbonate buffer (pH 9.4) were preincubated for 5 min at 25 °C, and then PGO (freshly made up 50 mM stock solution in water) was added to a final concentration of 0.5–4.0 mM. Aliquots were removed at various times for enzyme assay.

Inactivation with Tetranitromethane (TNM)—Type II DHQase was preincubated in 0.1M Tris/HCl (pH 8.0) for 5 min at 25 °C, and then TNM (freshly made up stock solution of 15 mM in 95% ethanol) was added to a final concentration of 0.5–4.0 mM. Aliquots were removed at various times for enzyme assay.

Preparation of Inactivated Enzyme Samples for ES-MS—Enzyme inactivated to different extents was prepared for mass spectrometry by stopping the reaction by gel filtration on a Sephadex G-50 column in 0.1% (v/v) trifluoroacetic acid was applied to elute the peptides. The column eluent was introduced directly into the mass spectrometer with a pneumatically assisted electro-spray ion source (VG Biotech Ltd., Altrincham, Cheshire, UK). Carrier solvent (1:1 (v/v) acetonitrile/water, 0.2% formic acid) infusion was controlled at 10 μl/min using a Harvard syringe pump (Harvard Apparatus, South Natice, MA). Protein samples were dissolved in carrier solvent at a concentration of 20 pmol/μl, centrifuged at 5000 × g for 2 min, and then 10–20 μl samples injected directly into the carrier stream. Maximum entropy deconvolution (27) was applied for quantitative analysis of the raw data using a 1.0-Da peak width and 1.0-Da/channel resolution.

The protein digests were separated by HPLC on a C-4 reverse phase column (2.0 × 150 mm; Delta-pac C18, Waters, Waterford, Hertfordshire, UK) using 2% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid as the initial solvent (flow rate 0.25 ml/min); after an 8-min wash to remove guanidinium hydrochloride a linear gradient of 2–70% acetonitrile (v/v) in 0.1% (v/v) trifluoroacetic acid was applied to elute the peptides. The column eluent was introduced directly into the mass spectrometer with a dry nitrogen flow of 400 liters/h, and the source temperature was set at 100 °C. The absorption profile of the eluted peptides was recorded at 214 nm, and centroid mass spectra in the range 400-1800 Da were recorded at 4-s intervals.

Site-directed Mutagenesis—The plasmid containing the coding sequence of S. coelicolor DHQase cloned into the Smal site of pBluescribe (Eastman Kodak Co.); (pDHQ) was a gift from Professor Ian S. Hunter, University of Strathclyde, Glasgow. Three site-directed mutants (R23K, R23Q, R23A) were prepared using polymerase chain reaction site-directed mutagenesis (28). Primers were synthesized on an Applied Biosystems Model 280A DNA synthesizer. The three reverse polymerase chain reaction primers that contained the mismatch codon (highlighted in bold) overlap the BglII site that is located 10 base pairs downstream of arginine residue 23, 5'-TGATACCTGCCGCTGCTTCGTGCCGACGTCGAGATCTCCGGCGCCTCCGGCAGC (R23K): 5'-TGATACCTGCCGCTGCTGCCTGCCGAGC3' (R23Q): 5'-TGATACCTGCCGCTGCTGCCTGCCGAGC3' (R23A); 5'-TGATACCTGCCGCTGCTGCCTGCCGAGC3' (R23Q). The forward-primer was complementary to a region of the polylinker 190 base pairs upstream and contained an EcoRV site: 5'-CCAGATATCAATATACGACTATAGG3' (239). The polymerase chain reaction products were cloned back into pDHQ replacing the wild-type BglII-EcoRV fragment. The presence of only the desired mutations was confirmed by DNA sequencing on both strands (29).

Circular Dichroism—The circular dichroism (CD) spectra of each protein were recorded in a Jasco J-600 spectropolarimeter. Spectra in the far UV region (260–190 nm) were recorded in cylindrical quartz cells of path length 0.02 cm, and spectra in the near UV region (320–260 nm) were recorded in cells of path length 0.5 cm. The protein

3 P. J. White, G. Young, T. Krell, I. S. Hunter, and J. R. Coggins, manuscript in preparation.

4 W. Gilbert (1991) unpublished information, obtained from VecBase 3.0, copyright IBI.
Fig. 2. Sequence alignment of currently available type II and monofunctional type I DHQases. Boxed amino acids form the common motif. Highlighted are the hyper-reactive arginine residues and the essential tyrosine.

Fig. 3. Reverse phase chromatography/electrospray mass spectrometry data of a tryptic digest of E. coli DHQase. 20% inactivated after phenylglyoxal treatment. The enzyme sample is a mixture of 50% native enzyme and 20% single site-modified enzyme; centroid spectra were collected every 4 s. Traces A, B, and C show the relative abundance of mass species in all the recorded spectra against retention time. A, scan within all the recorded spectra for a mass of modified peptide 208–213 + 116 Da (1 PGO attached); B, scan for a mass of unmodified peptide 214–229; C, scan for the mass of unmodified peptide 208–213 (the double peak in this trace is due to peptide 26–31 that has exactly the same mass as peptide 208–213; these two modified peptides were only minor species, whereas the third peak size on the HPLC trace it was apparent that two of these species at Arg-213 (Fig. 3) demonstrate that the protease cuts the unmodified enzyme inactivated after phenylglyoxal treatment. A minor peak that corresponds to an oxidized species could also be detected. To localize the site of modification a 30% inactivated sample was digested with trypsin. A peptide with the mass of 2096.3 Da could be detected that was not present in the tryptic digest of the native enzyme (data not shown). This mass corresponds to peptide 24–42 + 45 Da. The sequence contains only one tyrosine residue (Tyr-28) that is conserved in all the type II DHQases (Fig. 2).

Identification of an Essential Tyrosine Residue (Tyr-28) in the Type II DHQase of S. coelicolor—S. coelicolor DHQase can be inactivated by treatment with the nitrating reagent TNM. This reagent is reasonably specific for tyrosine residues (35). Inactivation followed pseudo-first order kinetics (data not shown). The nitration of the aromatic ring of a tyrosine residue results in a mass increase of +45 Da. Enzyme inactivated to different extents showed only one modified tyrosine residue in the mass spectrometer (Fig. 1, exemplified by 30 and 70% inactivated species). A minor peak that corresponds to an oxidized species could also be detected. To localize the site of modification a 30% inactivated sample was digested with trypsin. A peptide with the mass of 2096.3 Da could be detected that was not present in the tryptic digest of the native enzyme (data not shown). This mass corresponds to peptide 24–42 + 45 Da. The sequence contains only one tyrosine residue (Tyr-28) that is conserved in all the type II DHQases (Fig. 2).

Identification of a Hyper-reactive Arginine (Arg-213) in the Type I DHQase of E. coli—The type I DHQases, like the type II enzymes, can be inactivated by PGO; in the case of the E. coli enzyme the inactivation follows pseudo-first order kinetics (data not shown). 20% inactivated enzyme showed only one modified species with a mass difference of +116 Da that corresponds to the incorporation of one PGO per reacting site. Native and 20% inactivated enzyme were digested with trypsin, and peptides were separated by HPLC and injected directly into the electrospray mass spectrometer. Trypsin cleaves at unmodified arginine and lysine residues; no cleavage is expected after a modified arginine residue. Recorded spectra were therefore scanned for the theoretical tryptic peptides containing a single internal PGO-modified arginine residue (+116 Da). This tryptic digestion allows the precise location of the site of reaction. Three modified peptides were detected. From the peak size on the HPLC trace it was apparent that two of these modified peptides were only minor species, whereas the third modified peptide was much more abundant. This abundant arginine-containing peptide corresponds to residues 208–213 (Fig. 3A) and identifies Arg-213 as the hyper-reactive arginine residue (Fig. 2). Peptides 208–213 and 214–229 are shown to respond to the incorporation of one PGO per reacting site. For the Type II DHQase of S. coelicolor, Arg-213 is the hyper-reactive arginine residue (Fig. 3B and 3C). The double peak in Fig. 3C is due to the occurrence in the tryptic digest of a second peptide...
(26–31) that has exactly the same mass as peptide 208–213; these peptides have different mobilities on reverse phase HPLC. The secondary sites of modification are at residues 38 and 48. The tryptic digest of native enzyme did not contain species with masses corresponding to the PGO-modified peptides.

Site-directed Mutagenesis of the Hyper-reactive Arginine Residue in the Type II DHQase of S. coelicolor—The hyper-reactive arginine (Arg-23) of S. coelicolor DHQase (21) was replaced by a lysine, glutamine, and alanine residue. The masses of purified mutant proteins were determined using ES-MS. All three masses were found to be very close to the theoretical values: 16,467.00 ± 1.55 Da for R23A (theoretically 16,465.56 Da), 16,522.79 ± 1.18 Da for R23Q (theoretically 16,522.61 Da), and 16,523.87 ± 2.33 Da for R23K (theoretically 16,522.66 Da).

Evidence from far UV CD spectroscopy (Fig. 4A, Table I) demonstrates that the secondary structure of the protein was not altered significantly by the mutations. Only the R23Q mutation lead to a small detectable change in the β-sheet content (Table I). The near UV CD spectra (Fig. 4B) also confirm that the tertiary structures of the mutant enzymes are very similar to the wild-type, although there are some small changes.

**Table I**

|          | α-Helix | β-Sheet | Remainder |
|----------|---------|---------|-----------|
| Wild-type| 21 ± 1.1| 41 ± 1.1| 39 ± 2.0  |
| R23K     | 20 ± 0.8| 40 ± 0.9| 40 ± 1.6  |
| R23Q     | 21 ± 0.9| 35 ± 1.1| 44 ± 1.8  |
| R23A     | 22 ± 1.2| 41 ± 1.3| 36 ± 2.3  |
differences in the 270–285 nm region suggesting a subtle change in the environment of one or more tyrosine side chains.

The kinetic parameters were determined as shown in Table II. The \( K_m \) values were calculated using a Lineweaver-Burk plot, and the mutant enzyme activity was determined as described above for the wild-type enzyme. Surprisingly, the \( K_m \) values of the mutant enzymes were found to be lower than those of the wild-type enzyme. This apparent increased affinity for substrate was confirmed by isothermal titration experiments of enzyme species with an equilibrated substrate/product mixture as illustrated in Fig. 5 and Table III. With wild-type enzyme the heat effects were relatively small but, after correction for dilution heat effects, consistent with weak exothermic 1:1 enzyme-substrate complex formation (\( K_{app} \approx 5 \text{ mM} \), see Table III). In marked contrast, addition of substrate to mutant proteins gave significantly more exothermic initial heat effects, which decreased rapidly with subsequent injections in a manner consistent with much tighter binding (\( K_{app} \approx 0.3–1 \text{ mM} \); Fig. 5B and Table III).

**DISCUSSION**

Both chemical modification and site-directed mutagenesis of the conserved hyper-reactive arginine residue in the type II DHQases (Arg-23 in the *S. coelicolor* enzyme) lead to loss of enzyme activity. Arginine residues with hyper-reactivity toward \( \alpha \)-1,2-dicarbonyls such as PGO are often found to be involved in carboxylate binding. Such residues are located in special microenvironments and have lower \( pK_a \) values than other arginine residues (36, 37). Three mutants were made of the hyper-reactive Arg-23 of *S. coelicolor* DHQase, R23K, R23Q, and R23A. Mass spectrometry and CD analysis revealed that the mutants were of the expected size and had the same or very similar secondary structure as the wild-type enzyme (Fig. 4, Table I). All three mutants showed a much smaller turnover number (\( k_{cat} \)), with R23K being in the same range as R23Q and with R23A still being another order of magnitude less active (Table II). In contrast a decrease in \( K_m \) values was observed, by a factor of 4 for the R23K mutant and by factors of 8 and 6 for the R23Q and R23A proteins, respectively (Table II). This behavior was also confirmed more directly by calorimetric binding studies, which showed that binding of substrate was significantly enhanced by replacement of Arg-23 with Lys, Gln, or Ala, as indicated by a 5–10-fold decrease in the apparent binding constant (\( K_{app} \)) and significantly more exothermic binding. Direct comparison of \( K_{app} \) with \( K_m \) values is not strictly appropriate because \( K_m \) values relate to initial substrate binding rather than the equilibrated substrate/product mixture used for calorimetric experiments. They are, in any case, only related to substrate binding affinities within the validity of the Michaelis-Menten approximation. Nevertheless, \( K_m \) and \( K_{app} \) are of the same order of magnitude and, more importantly, show the same trends in the mutant enzyme.

In other enzymes, where arginine residues are known to be involved in the recognition of substrates containing carboxyl groups, arginine mutation leads to a large increase in \( K_m \) (25, 38). Our results therefore suggest that Arg-23 is not simply

| \( k_{cat} \) (s\(^{-1}\)) | \( K_m \) (\( \mu \text{M} \)) | \( k_{cat}/K_m \) (s\(^{-1}\) M\(^{-1}\)) |
|----------------|----------|------------------|
| Wild-type     | 960      | 1100             | 8.7 \times 10^5  |
| R23K          | 0.35     | 250              | 1400             |
| R23Q          | 0.31     | 135              | 2500             |
| R23A          | 0.032    | 170              | 188              |

**TABLE III** Apparent DHQase-substrate binding parameters of wild-type and mutant *S. coelicolor* DHQase determined from calorimetric titrations assuming 1:1 complex formation

| \( K_{app} \) (M) | \( \Delta H_{app} \) (kJ mol\(^{-1}\)) |
|------------------|-------------------------------------|
| Wild-type        | 5.40                                |
| R23K             | 1.10                                |
| R23Q             | 0.33                                |
| R23A             | 0.30                                |
involved in substrate recognition but must have a catalytic role. One possibility is that a positive charge is required to stabilize a negative transition state such as an enolate. Although the R23K mutant retains the positive charge, the position of this charge will be significantly altered in the mutant. The full interpretation of this result must await the determination of the three-dimensional structure of the enzyme.

Chemical modification has also identified Tyr-28 as a residue in or near the active site. Both Arg-23 and Tyr-28 are conserved in all type II DHQases (Fig. 2). The small changes observed in the near UV CD spectra (Fig. 4B) of the arginine mutant proteins can be explained by a change in the environment of a tyrosine residue. The proximity in the sequence of Arg-23 and Tyr-28 and the occurrence of these small spectral changes when the arginine is altered are both consistent with these residues being spatially close.

Chemical modification experiments on the type I DHQases have also identified a hyper-reactive arginine (Arg-213 in the E. coli enzyme) that appears to be essential for activity and is near the methionine residue previously identified as an active site residue (17). Comparison of the amino acid sequences around the hyper-reactive arginine residues of the two classes of enzyme (Fig. 2) indicates that there is a conserved structural motif, extending over 9 residues, shown by the shaded region in Fig. 2. In the type I enzymes the essential tyrosine residue identified by TNM modification in the type II enzymes is replaced by a phenylalanine residue. Both the mechanistic experiments (3, 13) and the sequence comparisons (11, 30) have suggested that these two classes of enzyme are likely to be structurally very different, and it will therefore be interesting to see whether this sequence similarity is reflected in a common substrate binding fold at the active centers of these two classes of enzyme.

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