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HYBRID FORMATION IN THE SHIKIMATE PATHWAY ENZYME DEHYDROQUINASE

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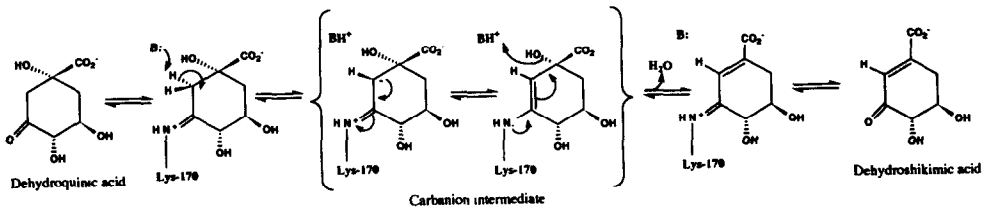
Abstract
The guanidine hydrochloride-induced denaturation of unmodified and product-linked forms of the dimeric shikimate pathway enzyme 3-dehydroquinase are shown, by fluorescence spectroscopy, to be reversible. Mixing the unfolded polypeptides prior to renaturation yields a hybrid dimer, which has been identified by isoelectric focussing and its relative yield quantitated by laser densitometry.

3-Dehydroquinase (dehydroquinate dehydratase; DHQase) catalyses the conversion of 3-dehydroquinate to 3-dehydroshikimate (Scheme 1). Two types of DHQase are known, type I and type II, which have very different primary sequences, subunit molecular weights, secondary structure compositions, oligomeric structures, thermal stabilities and catalytic mechanisms[1]. 3-Dehydroquinase activity is associated with two metabolic pathways; the biosynthetic shikimate pathway, which is ubiquitous in both prokaryotes and the lower eukaryotes, and the catabolic quinate pathway which appears to be specific to fungi, such as *Neurospora crassa* and *Aspergillus nidulans*[2-4]. Type I enzymes have only been found in the context of the shikimate pathway whereas type II enzymes have been found in both pathways[5, 6].

The subject of this communication is 3-dehydroquinase (M_r = 27,466) from the shikimate pathway of *E.coli* (EC4.2.1.10). This typical type I enzyme has been the focus of several recent structure/function investigations aimed at elucidating its catalytic mechanism with a view to designing inhibitors which might act as antimicrobial agents or herbicides. The enzyme has been cloned, sequenced, overexpressed and purified to homogeneity[7-9]. Crystals of this enzyme have been grown but are not suitable for X-ray diffraction, however, crystals for another type I enzyme (that from *Salmonella typhi*) which is ~70% identical to the *E.coli* enzyme have yielded crystals suitable for X-ray diffraction studies[10]. The stereochemistry for the elimination reaction is known to be *syn*[11] and several active site residues have been identified by chemical modification studies[9, 12-14]. The mechanism that has emerged is shown in Scheme 1. A Schiff's base intermediate is formed between the substrate keto group and Lys-170 which acts as an electron sink[9], following the abstraction of the *pro-R* hydrogen at C2 by a



Scheme 1 Mechanism of the type I 3-dehydroquinase from *E.coli*.

postulated general base, His-143[14]. The hydroxyl group is then eliminated, possibly by general acid catalysis, resulting in the product Schiff's base which then breaks downs to yield the final product.

During the course of an investigation into the effects of ligand binding on the conformation of the *E.coli* type I enzyme it was discovered that reduction of the Schiff's base intermediate with sodium borohydride, to yield the secondary amine, resulted in a two-fold increase in the conformational stability of the enzyme and an increase of 40°C in its melting temperature[15]. It has since been found that only the product, dehydroshikimic acid, is covalently trapped at the enzyme active site by borohydride reduction[16], a possible consequence of the equilibrium constant for the reaction (K_{eq} = 15)[17]. The remarkable increase in the stability of *E.coli* DHQase induced by product linkage is of general interest especially with regard to the stability of enzymes in which covalent intermediates are involved, since the reduced form of the enzyme can be viewed as a stable derivative of the Schiff's base intermediate. Thus, a dissection of the biophysical mechanisms leading to this stabilization is of importance in the area of ligand-induced stabilization of proteins.

Hydrodynamic data have shown that the tremendous increase in conformational stability which results from trapping the product Schiff's base at the active site is mediated through the protein dimer, since the molecular weight and sedimentation coefficient of the modified dimer (which are identical to the unmodified enzyme under native conditions) remain almost unchanged at guanidine hydrochloride concentrations which denature the unmodified protein (Reilly, A., Morgan, P., Davis, K., Harding, S., Rowe, A., Coggins, J.R. & Kleanthous, C., unpublished observations). One route to further probing this effect, at the level of the individual subunits, is to create a hybrid dimer in which only one of the subunits has the product Schiff's base trapped at the active site while its partner remains unmodified. Thus, the effect of a singly liganded subunit on its unliganded partner can be investigated. In this communication we report a strategy for the formation of such a hybrid and experimental evidence verifying that this strategy does indeed yield a hybrid molecule.

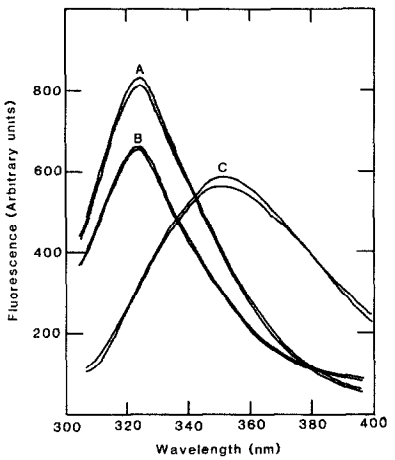


Fig. 1 Tryptophan fluorescence emission spectra of unmodified (A), borohydride-reduced (B) and Gn.HCl-denatured (C) forms of *E.coli* dehydroquinase. The two curves in each of A and B show native and refolded enzyme which are essentially indistinguishable in each case, while the two curves in C show that the denatured forms of both unmodified, A, and borohydride-reduced DHQase, B, are very similar[19].

Making a hybrid DHQase dimer requires that subunits from native, unmodified enzyme are mixed with those of fully modified protein and hybrids allowed to form. As no method exists for the separation of DHQase subunits under native conditions we chose to perform the experiment under denaturing conditions and then renature the mixture. Whereas a great deal is known about the denaturation of DHQase[15] very little is known concerning its renaturation, especially for the borohydride-reduced enzyme. Therefore, the renaturation of Gn.HCl-denatured, unmodified and borohydride-reduced DHQases were analysed by fluorescence spectroscopy, a technique sensitive to the folded state of a protein[18]. The fluorescence spectrum for native DHQase, which has a single tryptophan residue and which has not been reported previously, has an emission maximum at 322nm (Fig.1, A)[19]. This value is highly blue-shifted from that of free tryptophan (~350nm) which indicates that the single tryptophan of the enzyme is buried in the interior of the protein[18]. Borohydride reduction of the product Schiff's base quenches this signal by ~20% (Fig.1, B). When both proteins are denatured in 6M Gn.HCl the emission maxima for both proteins superimpose and shift to 354nm (Fig.1, C), consistent with the signal from a tryptophan in an unfolded polypeptide. Renaturation of both enzymes was accomplished by a ten-fold dilution of the denaturant. In both cases emission curves almost superimposable with their native counterparts were obtained (Fig.1, A & B). Identical results have also been obtained by circular dichroism (Kelly, S., Price, N.C., Coggins, J.R. & Kleanthous, C, unpublished results). In addition, gel filtration experiments verified that the protein dimer had been recovered in both cases (data not shown).

The strategy for making a DHQase hybrid is shown in Fig. 2A. Since the renaturation experiments in Fig.1 showed that the native structure of both enzymic forms could be recovered simply by dilution of the denaturant, the strategy entailed mixing the two unfolded polypeptides prior to the renaturation step. The next problem to be addressed was the detection of such a hybrid. This was over-

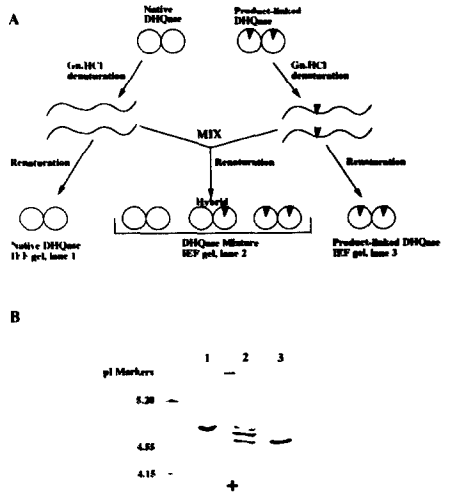


Fig. 2 Making and detecting a hybrid DHQase dimer. The strategy for making a hybrid DHQase is shown in the top part of the figure (A) while the Coomassie-stained isoelectric focussing gel (pH gradient 4-6.5) in the bottom part of the figure (B) provides the experimental verification that a hybrid has indeed been created[20]. The lanes are as described in 'A'.

come by capitalizing on the charge difference between the native and borohydride-reduced forms of DHQase; the product molecule has a single carboxylate group and so should increase the negative charge of the protein accordingly. Native polyacrylamide gels certainly indicated that the borohydride-reduced enzyme was more negatively charged than unmodified enzyme but the change in electrophoretic mobility was too small to allow the detection of a hybrid (unpublished observations). However, isoelectric focussing gels did provide the resolution required to detect, and eventually, quantitate such a species (Fig.2B).

The isoelectric point of native, refolded DHQase is 4.9 (Fig.2B, lane1)[20]. Borohydride-reduction of the product Schiff's base decreases the isoelectric point of the refolded protein to 4.6 (Fig.2B, lane 3), consistent with the addition of the extra negative charge from the ligand. The refolded mixture shows the presence of both unmodified and modified protein but also an extra band which has a pI intermediate between these two forms, ~4.75 (Fig.2B, lane 2). The appearance of this band is fully consistent with it being a hybrid dimer of DHQase containing one native and one product-linked subunit.

The relative yield of hybrid was estimated by Coomassie staining a preparative IEF gel followed by laser densitometric scanning[21]. A typical example of such an experiment is shown in Fig. 3. The approximate ratio of unmodified:hybrid:modified DHQase obtained from such gels is 1:2:1, which is the anticipated ratio assuming the two types of DHQase still form the same subunit contacts.

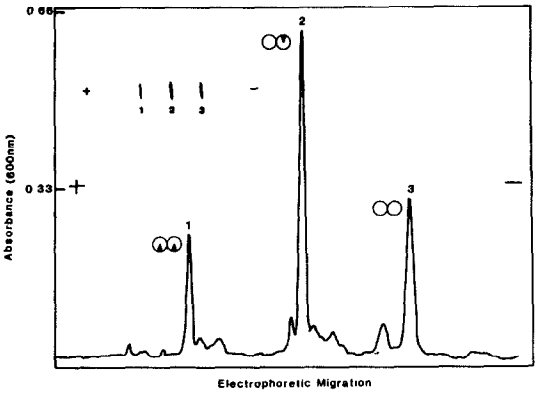


Fig. 3 Quantitation of relative hybrid yield by laser densitometry. A refolded hybrid mixture was electrophoresed in an isoelectric focussing gel (pH gradient 4-5) and stained with Coomassie blue[21]. The inset to the figure shows the electrophoretic pattern obtained. Laser densitometric scanning of these bands resulted in the absorbance pattern shown in the figure. The identity of each peak is given by the dimer structure indicated, which corresponds to the general scheme shown in Fig. 2.

In conclusion, we have shown, using fluorescence spectroscopy, that the denaturation of unmodified and product-linked DHQase is completely reversible by simple dilution of the denaturant. This has made possible a strategy to create a hybrid DHQase dimer in which one subunit contains the trapped, covalent intermediate while the other is free of ligand. The hybrid has been identified and its yield estimated by isoelectric focussing, a technique which will eventually be used to purify this species.

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19. Unmodified and borohydride reduced samples were prepared as described in ref.15. The protein concentration (determined using the molar absorption coefficient at 280nm of 0.69mL.mg^{-1}cm^{-1} [14].) was 7μg.mL^{-1} in each case. All samples were dissolved in potassium phosphate buffer (pH7.0, 50mM) containing dithiothreitol (1mM), except C which also contained Gn.HCl (6M), and made up to a volume of 2ml. Denatured samples (C) were renatured by 10-fold dilution into the same buffer without denaturant at 25°C, hence these samples contained 0.6M Gn.HCl. Fluorescence excitation was at 295nm and the buffer baseline was subtracted from each spectrum. All spectra were collected at 25°C.

20. Renatured samples were dialysed extensively against potassium phosphate buffer (pH7.0, 50mM, 4°C) containing dithiothreitol (1mM) prior to electrophoresis on pre-cast IEF gels (pH gradient 4-6.5) using a Pharmacia Phast system and stained with Coomassie Brilliant Blue.

21. To quantitate the relative ratios of each species following renaturation of the unfolded polypeptides, larger IEF gels (using a Pharmacia Multiphor system) were run over a narrower pH range (pH 4-5), stained with Coomassie Brilliant Blue and scanned using an LKB gel scanner.