## RESEARCH COMMUNICATION

# Evidence from kinetic isotope studies for an enolate intermediate in the mechanism of type II dehydroquinases

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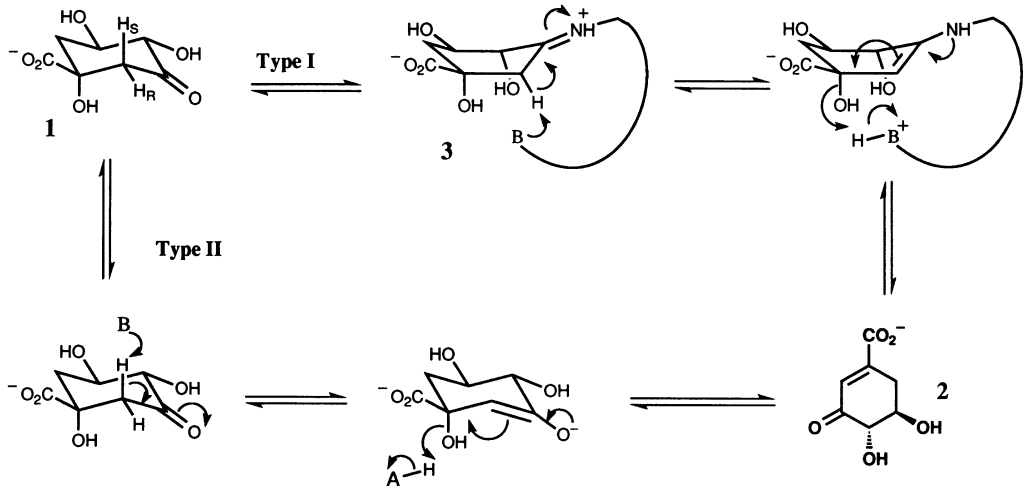
Substrate isotope studies, solvent isotope studies, proton inventories and studies of $V_{max}$ and $K_m$ as a function of pH suggest an $E_1CB$ (V. E. Anderson (1991) in Enzyme Mechanisms for Isotope Effects (Cook, P. F., ed.), pp. 389–417, CRC Press, Boca Raton, FL) mechanism via an enolate intermediate for type II dehydroquinases involved in biosynthetic or catabolic pathways.

## INTRODUCTION

The enzyme dehydroquinase (3-dehydroquinate dehydratase, EC 4.2.1.10) catalyses the reversible dehydration of dehydroquinate (**1**) to form dehydroshikimate (**2**) (Scheme 1). This reaction is a step in two metabolic pathways: the biosynthetic shikimate pathway and the catabolic quinate pathway [1–3]. Two structurally distinct classes of dehydroquinase are known (type I and type II), which possess different biochemical and biophysical properties and show no sequence similarity [4]. The type I enzymes appear to be restricted to the shikimate pathway, whereas the type II enzymes serve either biosynthetic or catabolic roles [4], or both [5].

The type I enzymes have a conserved lysine residue [6] and catalyse a *cis*-elimination via an imine intermediate (**3**). A conserved histidine residue has been identified as the general base [7]. The mechanism for the type I dehydroquinase is well characterized, and an enzyme-bound intermediate in the mechanism has been detected by electrospray MS [8]. In contrast, much less is known about the mechanism of the type II enzymes, which must be different because there are no conserved lysine residues and the enzymes are insensitive to treatment with substrate and sodium borohydride [4]. Preliminary crystallographic data on the type II enzyme from *Mycobacterium tuberculosis* have recently been published [9], and an essential active-site arginine residue has been identified in the enzymes from *Streptomyces coelicolor* and *Aspergillus nidulans* [10]. The most significant mechanistic result is that the type II enzymes carry out the elimination with the opposite stereochemistry, catalysing the *trans*-dehydration of dehydroquinate with loss of the more acidic axial *pro-S* hydrogen atom from C-2 [11,12].

The type II enzymes from the shikimate and quinate pathways have significantly different steady-state kinetic parameters. The shikimate pathway type II dehydroquinase from *M. tuberculosis* [13] has a low $k_{cat}$ of 4 $s^{-1}$ and a low $K_m$ of 9 $\mu$M. These values were determined at pH 7 and at 25 °C, and compare with a $K_m$ of 64 $\mu$M determined at pH 7.2 and 37 °C [14]. The corresponding values for the *Escherichia coli* type I enzyme operating on the shikimate pathway are a $k_{cat}$ of 135 $s^{-1}$ and a $K_m$ of 16 $\mu$M [4]. The inducible quinate-pathway type-II enzyme from *A. nidulans* has a higher $k_{cat}$ of 1300 $s^{-1}$ and higher $K_m$ of 150 $\mu$M [4]. These differences presumably reflect the need for the inducible enzyme




**Scheme 1 Proposed mechanisms for the enzymic conversion of dehydroquinate (1) into dehydroshikimate (2) catalysed by type I (*E. coli* ) and type II (*M. tuberculosis* and *A. nidulans*) dehydroquinases**

to have a rapid turnover at times of high quinate availability. In the present paper we report the first systematic study of the mechanism of the type II dehydroquinases using a combination of primary $^2H$ substrate isotope effects, solvent isotope effects, proton inventory studies and pH-dependency of $V_{max}$ and $K_m$. Corresponding studies on the type I dehydroquinase from *E. coli* are also reported. The experiments suggest that the type II enzymes catalyse an $E_1CB$ [14a] elimination reaction via an enolate intermediate.

## METHODS

All chemicals and biochemicals were of the highest grade available and, unless otherwise stated, were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Milli-Q deionized water was used in all the buffers. The spectroscopic measurements were made on a Varian Cary 1E UV–visible spectrophotometer with a 1-cm-pathlength cell fitted with a Grant LTD6 thermostatically controlled water bath.

### Assay conditions

The reaction was monitored by observing the increase in absorbance at 234 nm in the UV spectrum due to product formation. Assays of type I dehydroquinase from *E. coli* were carried out in potassium phosphate buffer (50 mM, pH 7.0) at 25 °C. Assays of type II dehydroquinase from *M. tuberculosis* and *A. nidulans* were carried out in 10 mM Tris/acetate buffer at pH 7.0 and 25 °C. Kinetic constants were evaluated using the EnzFitter computer program.

### Substrate isotope effects

(*2R*)-[2-$^2$H]Dehydroquinate and (*2S*)-[2-$^2$H]dehydroquinate were prepared as described previously [12]. Solutions were calibrated by equilibration with dehydroquinase, and measurement of the change in the UV absorbance at 234 nm due to formation of the enone carboxylate chromophore of dehydroshikimate ($\epsilon_{234}$ $1.2 \times 10^4$ $M^{-1} \cdot cm^{-1}$). Calibrated solutions of (*2S*)-[2-$^2$H]dehydroquinate and non-$^2$H-labelled dehydroquinate were used as substrates for purified *A. nidulans* and *M. tuberculosis* type II dehydroquinases to determine any kinetic isotope effects on $V_{max}$ and $V_{max}/K_m$, while analogous experiments with (*2R*)-[2-$^2$H]dehydroquinate were used to determine the corresponding isotope effects with purified *E. coli* type I dehydroquinase [15].

### Solvent isotope effects

The solvent isotope effects on $V_{max}$ and $V_{max}/K_m$ were determined in $^1$H and $^2$H solutions at pH or p$^2$H 7.0 over a range of substrate concentrations. Separate stocks of phosphate buffer and dehydroquinate were made up by dissolving identical quantities of buffer salts/dehydroquinate in identical volumes of $H_2O$ and $^2H_2O$ [16].

### Proton inventory

The proton inventories were obtained by carrying out assays of the enzyme in mixtures of [$^2$H]- and [$^1$H]-phosphate buffer under $V_{max}$ conditions. Mixtures of $^1H_2O$ and $^2H_2O$ were prepared by volumetrically combining solutions at pH or p$^2$H 7.0. The difference in the density at 25 °C between $^2H_2$ and $H_2O$, and the isotopic dilution of the $^2H_2O$ solutions by protons released by the buffer salt were not taken into account when calculating the molar fraction of $^2$H present ($n$). This was simply calculated from the percentage of $^2H_2O$ present in each assay.

### pH-dependence of $V_{max}$ and $K_m$ for type II dehydroquinases

Studies were carried out at 25 °C at constant ionic strength ($I$0.01 M). For the pH range 6.0–9.0, Universal buffer A was used (5 mM acetic acid/5 mM Mes/10 mM Tris). For the pH range 8.5–10.5, Universal buffer B was used (10 mM Aces/5.2 mM ethanolamine/5.2 mM Tris). Both buffers were prepared immediately before use and the pH adjusted with tetramethylammonium hydroxide (Aldrich) for pH > 7 (buffer A) or pH < 7.8 (buffer B) or with acetic acid for pH 7 (buffer A) [17].

## RESULTS

The results of the substrate isotope studies and the solvent isotope studies carried out on the type I and two type II dehydroquinases are summarized in Table 1. Significant substrate isotope effects were apparent in direct measurements of $V_{max}$ using labelled and unlabelled dehydroquinate for both type II enzymes. The isotope effects on $V_{max}/K_m$ were measured using a range of substrate concentrations (from at least 0.5–6 $K_m$). The measured $(V_{max}/K_m)_{^1H}/(V_{max}/K_m)_{^2H} = 2.1 \pm 0.2$ for the *E. coli* type I enzyme compares with a corresponding $^3$H isotope effect of 2.3 reported by Rotenberg and Sprinson [18].

Solvent isotope effects were determined on $V_{max}$ and $V_{max}/K_m$ for each enzyme (Table 1) using unlabelled dehydroquinate in assays carried out in [$^2$H]buffer. The observed effects for the *A. nidulans* enzyme were quite large [$(V_{max}/K_m)_{^1H_2O}/(V_{max}/K_m)_{^2H_2O} = 2.5 \pm 0.5$, $(V_{max})_{^1H_2O}/(V_{max})_{^2H_2O} = 4.1 \pm 0.2$]. A smaller solvent isotope effect on $V_{max}$ was found with the *M. tuberculosis* enzyme ($2.48 \pm 0.05$) and, most significantly, no isotope effect on $V_{max}/K_m$ was observed. The solvent isotope effects for the type I dehydroquinase were small.

In order to gain information regarding the number of hydrogenic sites contributing to the observed solvent isotope effects on $V_{max}$ [16,19], the rates of reaction in buffers made with different mixtures of $^1H_2O$ and $^2H_2O$ were measured. Figure 1 shows plots of the observed rates against the molar fraction of $^2$H present in the solvent for the three enzymes. The straight-line relationship for the *M. tuberculosis* enzyme is consistent with a single proton contributing to the observed solvent isotope effect, whilst the bowl-shaped curve for the *A. nidulans* enzyme suggests the involvement of at least two protons. The dome shape of the proton inventory for the type I enzyme implies that there are offsetting normal and inverse contributions to the solvent isotope effect on $V_{max}$ and does not allow for determination of the number of hydrogenic sites participating.

The studies on type II dehydroquinases were extended to look at the variation of $k_{cat}$ and $K_m$ with pH over the pH range 6.0–10.5. Control experiments were performed to show that neither the equilibrium constant for the reaction nor the ab-

**Table 1 Summary of observed isotope effects on type I and type II dehydroquinases**

Results for $V_{max}$ are means ± S.D. for 3 observations. The errors for $K_m$ and $K_{max}$ were obtained from Enzfitter®. Errors quoted on combinations of parameters were calculated by addition in quadrature of the percentage errors of the two components.

| Parameter | Type II (*M. tuberculosis*) | Type II (*A. nidulans*) | Type I (*E. coli*) |
|---|---|---|---|
| $(V_{max})_{^1H}/(V_{max})_{^2H}$ | $2.80 \pm 0.08$ | $1.8 \pm 0.2$ | $1.28 \pm 0.05$ |
| $(V_{max}/K_m)_{^1H}/(V_{max}/K_m)_{^2H}$ | $2.3 \pm 0.3$ | $1.5 \pm 0.2$ | $2.1 \pm 0.2$ |
| $(V_{max})_{^1H_2O}/(V_{max})_{^2H_2O}$ | $2.48 \pm 0.05$ | $4.1 \pm 0.2$ | $1.64 \pm 0.04$ |
| $(V_{max}/K_m)_{^1H_2O}/(V_{max}/K_m)_{^2H_2O}$ | $1.0 \pm 0.1$ | $2.5 \pm 0.5$ | $1.4 \pm 0.1$ |

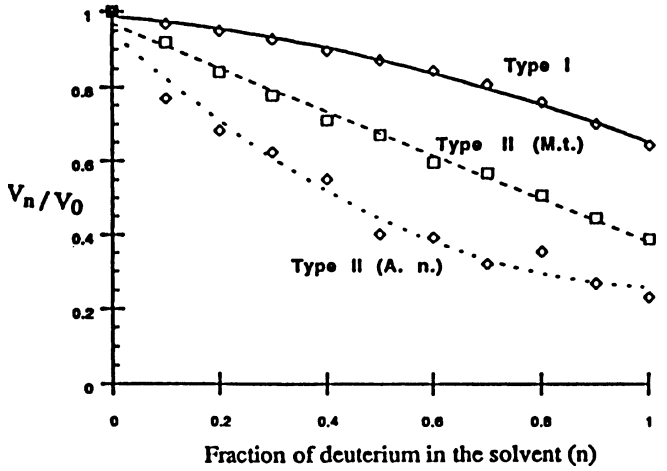



**Figure 1 Proton inventories for the reactions catalysed by *E. coli* dehydroquinase (type I) (–◇–), *M. tuberculosis* dehydroquinase (type II, biosynthetic) (- -□- -) and *A. nidulans* dehydroquinase (type II, catabolic) (· · · ◇ · · ·)**

$V_n$ is the rate of reaction when the fraction of $^2H$ in the solvent is $n$; $V_0$ is the rate when $n = 0$. The curves were fitted by using the computer program Kaleidograph.

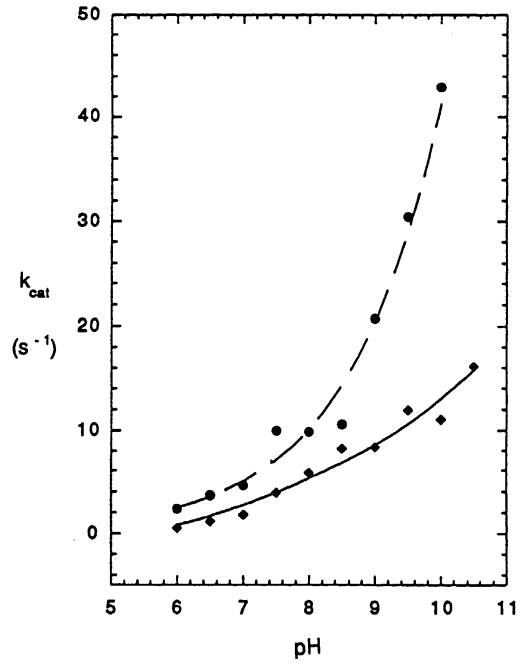



**Figure 2 Variation of $k_{cat}$ with pH for type II dehydroquinases from *M. tuberculosis* (◆) and *A. nidulans* (●)**

The curves were fitted by using the program Kaleidograph.

sorption coefficient for the product dehydroshikimate changed with pH over the range pH 6.0–10.5. For the *A. nidulans* enzyme, $k_{cat}$ (Figure 2) shows a steep increase above pH 8, which was still increasing at pH 10.5. A similar, but less dramatic, increase in $k_{cat}$ was observed for the slower *M. tuberculosis* enzyme. Because the conversion of dehydroquinate (**1**) into dehydroshikimate (**2**) can be catalysed by base in the absence of enzyme, the corresponding non-enzymic reaction was also studied (results not shown). This reaction was shown to contribute less than 1% to the rate of the slowest enzyme-catalysed reaction even at pH 10.5.

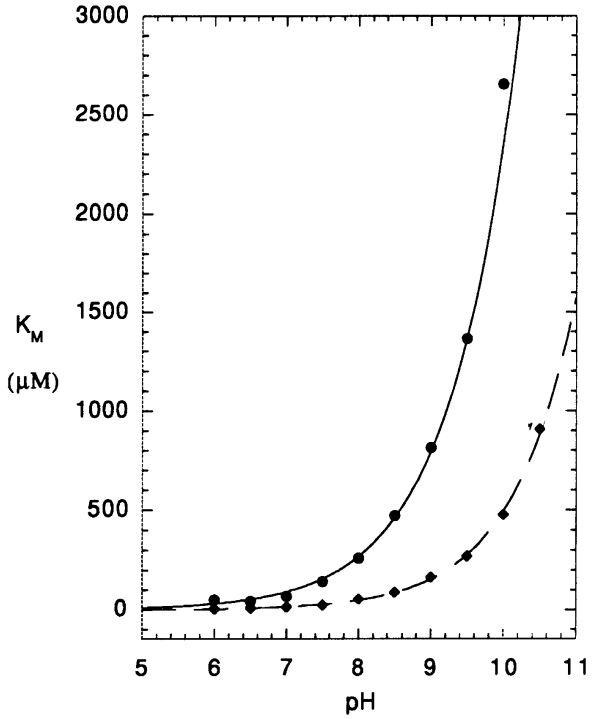



**Figure 3 Variation of $K_m$ with pH for type II dehydroquinases from *M. tuberculosis* (◆) and *A. nidulans* (●)**

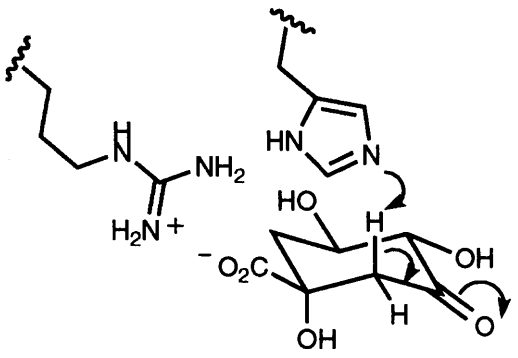
The curves were fitted by using the program Kaleidograph.

The variation of $K_m$ (Figure 3) with pH for the *A. nidulans* enzyme closely mirrors the pH-dependence of $k_{cat}$ (so much so that the changes effectively cancel out and $k_{cat}/K_m$ decreases by less than a factor of 4 between pH 6.0 and 10.5). A similar increase in $K_m$ above pH 9 is observed for the *M. tuberculosis* enzyme.

## DISCUSSION

The substrate and solvent isotope effects show similarities and differences between the type I and type II enzymes and also between the two type II enzymes. For all three enzymes the proton abstraction is partially rate-determining and occurs at or before the highest transition state. The substrate and solvent isotope effects measured for the type I enzyme are all small, consistent with a complex multistep mechanism where the steps involving proton transfer are not significantly slower than other steps in the mechanism (including perhaps product release). The proton inventory similarly cannot be interpreted in terms of a single simple transition state.

In comparing the slower biosynthetic *M. tuberculosis* type II dehydroquinase with the catabolic enzyme from *A. nidulans*, the most interesting and informative results are (i) the absence of a solvent isotope effect on $V_{max}/K_m$ for the *M. tuberculosis* enzyme, and (ii) the differences in the proton inventories for the two enzymes. The absence of $V_{max}/K_m$ solvent isotope effect for the *M. tuberculosis* enzyme indicates that the step which contributes to the solvent isotope effect on $V_{max}$ occurs after the highest point



**Scheme 2 Proposed detail of the active-site structure of type II dehydroquinases showing how an active-site arginine may influence the deprotonation and binding**

on the free-energy profile; molecules at this stage are already committed to catalysis. In contrast, the similarity of the isotope effects on $V_{max}$ and $V_{max}/K_m$ for the [$^2$H]substrate suggests that the removal of this $^2$H does occur at the transition state which is highest in energy.

Several of the possible mechanisms for the type-II-dehydroquinase-catalysed elimination are not consistent with the accumulated data. The absence of a solvent isotope effect on $V_{max}/K_m$ for the *M. tuberculosis* enzyme is not consistent with either a concerted E2 mechanism or a formation of an enol intermediate, both of which would be expected to involve a solvent-derived proton. A mechanism involving an enol would also not be expected to show the observed increase of $k_{cat}$ with pH. All the results for both type II enzymes are, however, consistent with a stepwise $E_1CB$ mechanism involving an enolate intermediate (**3**), formation of which is partially rate-limiting (Scheme 1).

The smaller substrate isotope effects, larger solvent isotope effects and enhanced rate observed for the *A. nidulans* enzyme compared with the *M. tuberculosis* enzyme are consistent with the relative energy of the transition state for the proton abstraction being reduced. This makes it less rate-determining than the subsequent solvent-dependent hydroxyl removal, which occurs at the highest point in the free-energy profile. The difference in the proton inventories for the *M. tuberculosis* and the *A. nidulans* type II enzymes can be interpreted as corresponding to the different numbers of hydrogen atoms contributing to the kinetically significant transition states. For the *M. tuberculosis* enzyme this suggests that there is a single exchangeable proton associated with the elimination step, whereas for the *A. nidulans* enzyme either two protons are involved in a single step or both the deprotonation and the subsequent elimination step, assumed to involve general acid catalysis, could possibly contribute a proton.

Both type II enzymes show a pH-dependence for $K_m$ which increases sharply above pH 9. There is corresponding increase in $k_{cat}$, especially for the *A. nidulans* enzyme, which follows a very similar ionization curve to $K_m$. Scheme 2 shows a proposal as to how these effects may arise. There is evidence for an arginine [10] and a histidine [4] residue in the active site of type II enzymes. Electrostatic interaction of the arginine with the histidine would moderate its basicity. As the arginine is deprotonated, it would bind the substrate less well thereby increasing $K_m$. However, the loss of the electrostatic interaction may also be expected to enhance the basicity of the enzymic base, leading to an increase in $k_{cat}$.

In conclusion, this series of kinetic experiments are consistent with the type II dehydroquinases catalysing an overall *anti* elimination by a stepwise $E_1CB$ mechanism involving an enolate intermediate (Scheme 1). Two further proposals come out of this work: firstly that the pH-dependence of $k_{cat}$ and $K_m$ may result from direct and indirect effects of deprotonating an active-site arginine, and secondly that the enhanced catalytic rate of the catabolic *A. nidulans* enzyme relative to the slower biosynthetic *M. tuberculosis* enzyme can be explained by greater stabilization of the transition state leading to the intermediate enolate.

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