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Mechanistic Studies on Type I and Type II Dehydroquinase with (6*R*)- and (6*S*)-6-Fluoro-3-dehydroquinic Acids

Emily J. Parker, [a,†] Concepción González Bello, [a,‡] John R. Coggins, [b] Alastair R. Hawkins [c] and Chris Abell [a,*]

[a]*University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK*
[b]*Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, UK*
[c]*Department of Biochemistry and Genetics, The Medical School, The University, Newcastle upon Tyne, NE2 7HH, UK*

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Abstract—(6*R*)- and (6*S*)-6-Fluoro-3-dehydroquinic acids are shown to be substrates for type I and type II dehydroquinases. Their differential reactivity provides insight into details of the reaction mechanism and enables a novel enzyme-substrate imine to be trapped on the type I enzyme. © 2000 Elsevier Science Ltd. All rights reserved.

The enzyme dehydroquinase (3-dehydroquinate dehydratase) catalyses the reversible dehydration of 3-dehydroquinic acid (**1**) to give 3-dehydroshikimic acid (**4**) (Scheme 1). This reaction is common to two metabolic pathways, the biosynthetic shikimate pathway, and the catabolic quinate pathway.[1] Two distinct classes of dehydroquinases (types I and II) are responsible for catalysing this transformation, by different mechanisms.[2] Type I dehydroquinases operate in the shikimate pathway, whereas type II enzymes have both biosynthetic and catabolic roles. The mechanism of the type I enzyme has been shown to involve imine formation[3] between the substrate and an active site lysine residue,[4] and involves loss of the *pro-R* hydrogen from C-2 of 3-dehydroquinic acid (**1**), corresponding to an overall *syn* elimination (Scheme 1).[5,6] In contrast the type II enzyme catalyses an *anti* elimination reaction with the loss of the more acidic *pro-S* hydrogen. It has been proposed that the reaction passes through an enolate intermediate.[7]

During the course of our work on fluorinated intermediates of the shikimate pathway[8] we prepared and purified (6*R*)- (**2**) and (6*S*)-6-fluoro-3-dehydroquinic acids (**3**). We now report our studies on the interaction of these compounds with type I enzyme from *Escherichia coli* and with both biosynthetic (*Mycobacterium tuberculosis*) and catabolic (*Aspergillus nidulans*) type II enzymes.[9–11]

Both 6-fluoro-3-dehydroquinic acids were found to be substrates for all three dehydroquinases. For all three enzymes the K_m values for (6*R*)-6-fluoro-3-dehydroquinic acid (**2**) were found to be lower than those determined for its (6*S*)-isomer **3** (Table 1). This difference is most pronounced for the type I enzyme. Both fluorinated substrates have lower K_m values for the type II enzymes than the natural substrate.

A trend in the k_{cat} values is also apparent. For all three enzymes the k_{cat} values show the same order of increase: (6*R*)-6-fluoro-3-dehydroquinic acid (**2**) < (6*S*)-6-fluoro-3-dehydroquinic acid (**3**) < 3-dehydroquinic acid (**1**). Of particular interest is the fact that the k_{cat} value for the (6*R*)-6-fluoro-3-dehydroquinic acid (**2**) is an order of magnitude lower than the corresponding value for (6*S*)-6-fluoro-3-dehydroquinic acid (**3**) for all three enzymes. These results show that not only does the presence of the fluorine slow down the enzymatic transformation, but that the stereochemical orientation of the fluorine has an effect.

Kinetic isotope effect studies have suggested that for the *M. tuberculosis* dehydroquinase, the proton abstraction

*Corresponding author. Tel.: +44-1223-336405; fax: +44-1223-336362; e-mail: ca26@cam.ac.uk
†Present address: Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand.
‡Present address: Departamento de Química Orgánica, Facultad de Química, Universidad de Santiago de Compostela, Santiago de Compostela, Spain.

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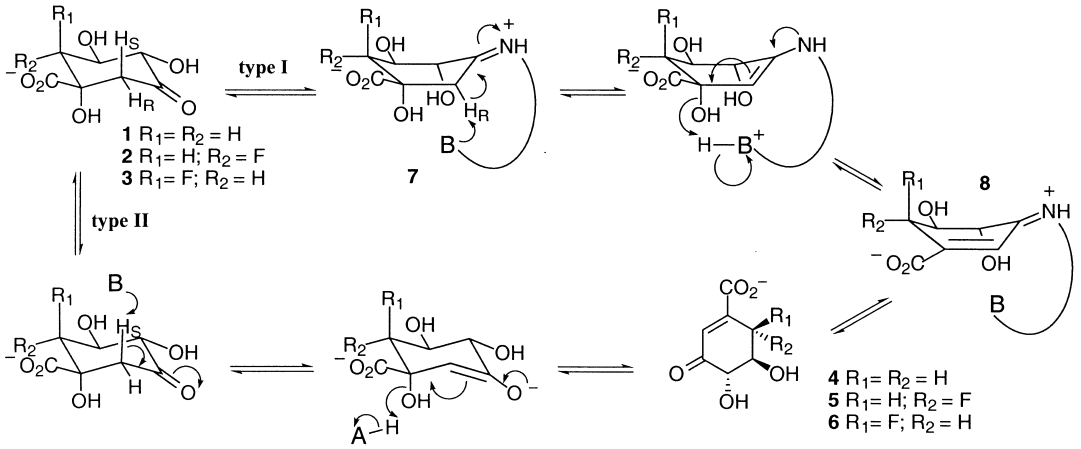


Scheme 1. Proposed mechanisms of types I and II dehydroquinases.

Table 1. Kinetic parameters for 3-dehydroquinic acid (**1**), (6*R*)-6-fluoro-3-dehydroquinic acid (**2**) and (6*S*)-6-fluoro-3-dehydroquinic acid (**3**) with types I and II dehydroquinases[a]

Source	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})×10^5
E. coli	**1**	18	158	8.8
(type I)	**2**	8.8	3.0	0.31
	3	120	36	0.31
M. tuberculosis	**1**	13	2.6	2.00
(anabolic type II)	**2**	3.6	0.14	0.39
	3	7.9	1.1	1.43
A. nidulans	**1**	122	313	25.7
(catabolic type II)	**2**	39	4.6	1.18
	3	50	46	9.2

[a]Assays were performed at substrate concentrations between 0.2 and 5 K_m, measuring initial rates at 234 nm for **1** ($\varepsilon = 1.2\times10^4$ M^{-1} cm^{-1}), 220 nm for **2** ($\varepsilon = 1.1\times10^4$ M^{-1} cm^{-1}) and 224 nm for **3** ($\varepsilon = 1.18\times10^4$ M^{-1} cm^{-1}). Assays were performed at 25 °C in potassium phosphate buffer (50 mM, pH 7.0) for the type I enzyme, and in Tris/HCl buffer (50 mM, pH 7.0) for the type II enzyme.

step is primarily rate determining (Scheme 1).[7] In contrast, for the *A. nidulans* dehydroquinase, the relative energy of the transition state for the proton abstraction step is reduced, increasing k_{cat} and making both the proton abstraction and hydroxyl removal steps partially rate determining. Introduction of a fluorine at C-6 of 3-dehydroquinic acid (**1**) is expected to disfavour the formation of the conjugated system in the hydroxyl removal step. The larger reduction in k_{cat} for the *A. nidulans* dehydroquinase with fluorinated substrates **2** and **3** is consistent with this idea, although the differential effects with these substrates for both type II enzymes suggests that the elimination step must now also be partially rate determining.

The equilibrium constant for the conversion of 3-dehydroquinic acid (**1**) to 3-dehydroshikimic acid (**4**) is 15.[12] ^{19}F NMR spectroscopy was used to determine the equilibrium for the dehydration of **2** and **3** (Fig. 1). For both 6-fluoro-3-dehydroquinic acids the equilibrium constant was reduced by unfavourable conjugation of the electronegative fluorine to the carbonyl, to 2.3 for (6*S*)-6-fluoro-3-dehydroquinic acid (**3**) and (6*R*)-6-fluoro-3-dehydroshikimic acid (**6**), and 0.7 for (6*R*)-6-fluoro-3-dehydroquinic acid (**2**) and (6*S*)-6-fluoro-3-dehydroshikimic acid (**5**). The smaller equilibrium constant for **2** (fluorine equatorial) and **5**, relative to **3** (axial fluorine) and **6**, suggests that destabilisation in the starting material due to the trans diaxial fluorine and hydroxyl in **3** is more important than effects due to the fluorine orientation in the product, which might be expected to be less favourable in **6** than **5**.

The reaction mechanism of the type I dehydroquinase involves imine intermediates between the enzyme and substrate (**7**) or product (**8**) (see Scheme 1). In principal, either can be trapped by treatment of the enzyme with sodium borohydride in the presence of an equilibrium mixture of substrate and product. However, only the product imine (**8**) was detected by electrospray mass spectrometry when the trapping experiment is carried out using an equilibrium mixture of 3-dehydroquinic acid (**1**) and 3-dehydroshikimic acid (**4**).[13] An analogous result was obtained when the trapping experiment was carried out in the presence of (6*R*)-6-fluoro-3-dehydroquinic acid (**2**) and (6*S*)-6-fluoro-3-dehydroshikimic acid (**5**).[14] The electrospray mass spectrum (ESMS) of an enzyme sample that had lost 30% of its activity shows the presence of modified protein (Fig. 2a) of molecular weight 27,644 ± 3. This is close to the predicted value of 27,641 for the reduced imine (**10**) between enzyme and 6-fluoro-3-dehydroshikimic acid (Scheme 2). In contrast, when the trapping experiment was repeated with (6*S*)-6-fluoro-3-dehydroquinic acid (**3**) and (6*R*)-6-fluoro-3-dehydroshikimic acid (**6**) the substrate imine is trapped. The ESMS of an enzyme sample that had lost 60% of its activity (Fig. 2b) shows a peak for modified protein at 27,657 ± 3, close to the expected mass of 27,659 for the reduced imine (**9**). This is the first (indirect) observation of the imine linked to substrate rather than product, and probably arises because the corresponding product imine complex is relatively destabilised by the axial fluorine.

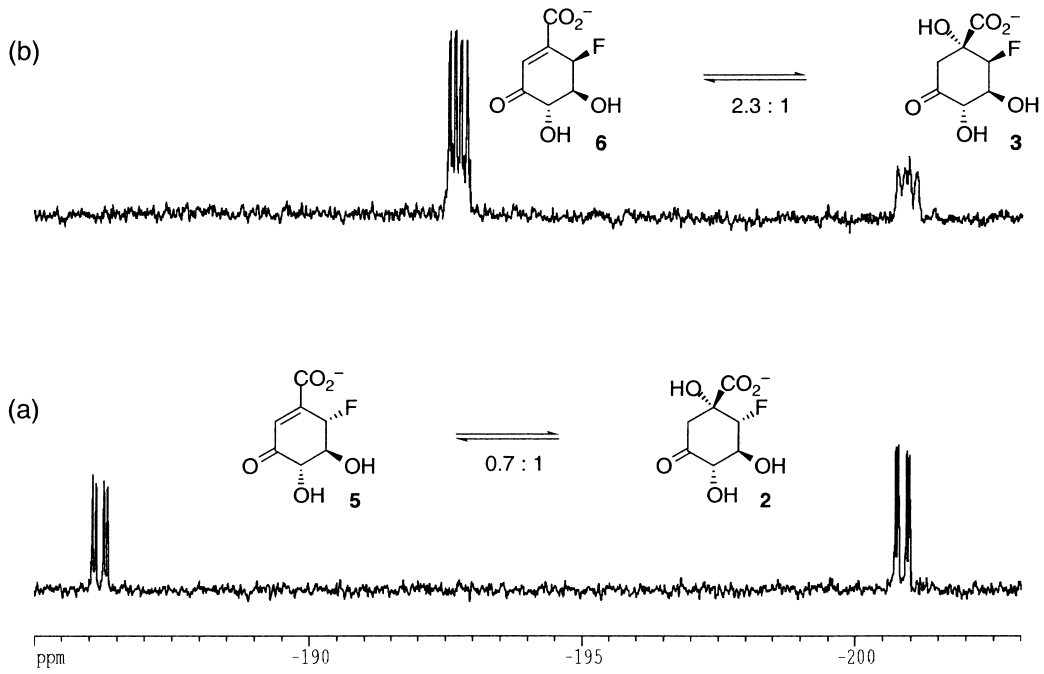


Figure 1. ^{19}F NMR spectra showing enzymatic equilibration of 6-fluoro-3-dehydroquinic and 6-fluoro-3-dehydroshikimic acids. ^{19}F NMR spectra (D_2O, 250 MHz) were recorded in potassium phosphate buffer (50 mM, pH 7.0) at 26 °C of an equilibrium mixture of: (a) (6*R*)-6-fluoro-3-dehydroquinic acid (**2**) and (6*S*)-6-fluoro-3-dehydroshikimic acid (**5**) in the presence of *E. coli* type I dehydroquinase (7 U). (b) (6*S*)-6-Fluoro-3-dehydroquinic acid (**3**) and (6*R*)-6-fluoro-3-dehydroshikimic acid (**6**) in the presence of *E. coli* type I dehydroquinase (0.5 U). Initial concentration of (6*R*)- **2** and (6*S*)-6-fluoro-3-dehydroquinic acids (**3**) was 2 mM.

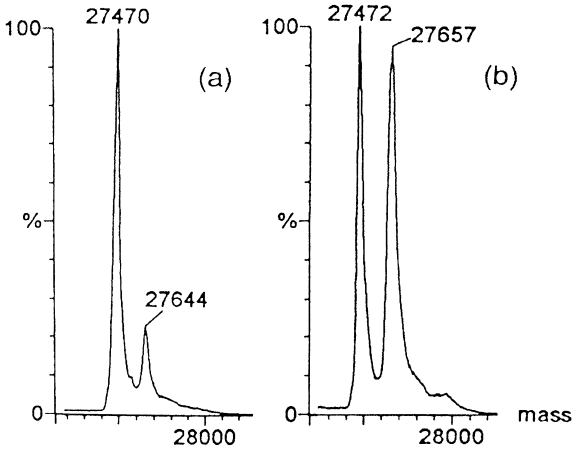
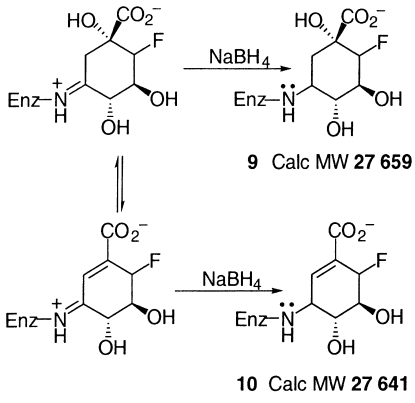


Figure 2. ESMS of *E. coli* type I dehydroquinase inactivated by treatment with sodium borohydride in the presence of an equilibrium mixture of (a) **2** and **5**, (b) **3** and **6**.



Scheme 2. Trapping enzyme–substrate imine intermediates with sodium borohydride on type I dehydroquinase.

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