# Heavy Atom Isotope Effects Reveal a Highly Polarized Transition State for Chorismate Mutase

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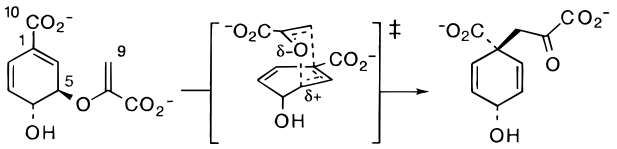
The conversion of chorismate (**1**) to prephenate (**2**) is catalyzed by the enzyme chorismate mutase.[1] The enzymatic reaction is formally a Claisen rearrangement, proceeding through a chairlike transition state,[2] but more detailed information on the structure of this high-energy species has remained elusive.

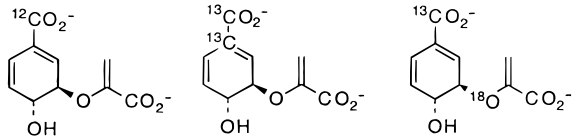


**1** **2**

Kinetic isotope effects (KIEs) are powerful tools in the determination of transition-state geometries. Secondary tritium isotope effects, for example, have shown that the uncatalyzed rearrangement of chorismate occurs via an asymmetric transition state in which the C(5)−O(5) bond is approximately 40% broken and the C(1)−C(9) bond is not detectably formed.[3] These results are in accord with calculations based on the RHF/6−31* transition structure for this reaction[4] and are typical for aliphatic Claisen rearrangements.[5,6] Unfortunately, no isotope effects were observed for the reaction catalyzed by the bifunctional chorismate mutase−prephenate dehydrogenase from *Escherichia coli*.[3] Suppression of the intrinsic isotope effect suggests a kinetically significant transition state prior to the rearrangement itself, presumably involving substrate complexation or a protein conformational change,[3] which nevertheless precludes direct study of the chemical reaction at the enzyme active site.

Viscosity variation experiments have shown that diffusive transition states only partially limit a second chorismate mutase, the monofunctional enzyme from *Bacillus subtilis* (BsCM).[7] Although BsCM and *E. coli* chorismate mutases have different tertiary folds,[8,9] they have comparable activities and inhibition profiles and similarly functionalized active sites.[9] Because highly active BsCM variants which are insensitive to viscosity are also available,[7] this is an excellent system for reexamining isotope effects on the enzymatic rearrangement of chorismate. Indeed, we have measured significant heavy atom effects on the reaction catalyzed by wild-type BsCM and the viscosity-insensitive C75S variant, showing that chemistry is largely rate determining and that the enzymatic transition state is highly polarized.

We used the sensitive remote label method[10] to measure primary heavy atom isotope effects at C(1) of chorismate, the site of bond formation, and at the ether oxygen, the site of bond cleavage. In these experiments, the carboxylate attached to C(1) served as the remote label, necessitating the synthesis of chorismate depleted in $^{13}C$ at this position (**1a**) and the doubly labeled derivatives **1b** and **1c**. The latter contain one heavy isotope at the indicator position and another at either C(1) (**1b**) or O(5) (**1c**).



**1a** **1b** **1c**

Substrates **1a**−**c** were prepared chemoenzymatically.[11] Established protocols[12,13] were adapted for the synthesis of labeled shikimates. Methyl shikimate depleted in $^{13}C$ at the carboxylate carbon was mixed with ca. 1.1% of appropriately double-labeled shikimate esters, and the resulting mixtures were saponified separately and converted biosynthetically to chorismate mixtures **1a**/**1b** and **1a**/**1c**. For the bioconversion, labeled shikimate was incubated with phosphoenolpyruvate and cell extracts from the previously described chorismate mutase-deficient *E. coli* strain KA12[14,15] harboring plasmid pKAD50,[16] which directs the overproduction of shikimate kinase, EPSP synthase, and chorismate synthase (the three enzymes on the biosynthetic pathway from shikimate to chorismate).

For determination of the isotope effects, unlabeled chorismate containing only the natural abundance of $^{13}C$ (Sigma) and the **1a**/**1b** and **1a**/**1c** substrate mixtures were converted enzymatically to prephenate (**2**) in separate experiments. In each case, three complete conversion reactions were performed at 2 mM chorismate with wild-type BsCM,[14] and three partial conversion reactions (to ca. 50%) were done for the wild-type enzyme and the C75S variant[7] at 4−6 mM chorismate. At the desired endpoint, the reaction was quenched with 5 M HCl. Prephenate decarboxylates under acidic conditions,[17] releasing the carbon at the indicator position as $CO_2$. Volatile products were collected under reduced pressure, using two pentane/liquid $N_2$ traps at −115 °C to remove $H_2O$ and other contaminants and a liquid $N_2$ trap at −196 °C to trap $CO_2$. The $^{12}CO_2/^{13}CO_2$ ratio of the sample was then determined on a Finnigan delta E isotope ratio mass spectrometer,

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**Table 1.** Kinetic Isotope Effects for Wild-Type BsCM and the C75S Variant[a]

| position (substrate) | KIE BsCM | KIE BsCM C75S | Becke3LYP 6-31G* |
|---|---|---|---|
| C(1) (**1a**/**1b**) | 1.0043 ± 0.0002 | 1.0057 ± 0.0002 | 1.0144 |
| O(5) (**1a**/**1c**) | 1.045 ± 0.003 | 1.053 ± 0.002 | 1.0432 |

[a] In 50 mM potassium phosphate buffer, pH 7.5, 22 °C. KIEs are corrected by division by the observed secondary kinetic isotope effect at C(10), which was measured to be 1.0009 ± 0.0001 for wild-type BsCM and 1.0016 ± 0.0002 for the C75S mutant.

and the heavy atom isotope effects were calculated by the equation

$$\mathrm{KIE} = \frac{\log(1 - f)}{\log(1 - f(R_p/R_0))} \quad (1)$$

where $R_0$ is the isotope ratio of the remote label in the starting material, $R_p$ is the isotope ratio of the remote label in the product, and $f$ is the fractional conversion of substrate to product.[18] The observed values for the **1a**/**1b** and **1a**/**1c** mixtures were divided by the secondary kinetic isotope effect at C(10), measured with unlabeled chorismate to give the desired $^{13}C$ and $^{18}O$ isotope effect.

The results are summarized in Table 1. We observe a very large $^{18}O$ effect on C−O bond cleavage and a smaller but still significant $^{13}C$ effect on C−C bond formation for the mutase-catalyzed reaction. Good evidence exists that product dissociation partially limits BsCM at saturation,[7,19,20] and the somewhat smaller isotope effects obtained here with the wild-type enzyme compared with the C75S variant would be consistent with substrate complexation contributing modestly to the overall barrier to reaction under *V/K* conditions as well. Nevertheless, the magnitude of the measured isotope effects leaves no doubt that, even for the wild-type enzyme, chemistry is significantly rate-determining.

The heavy atom isotope effects we obtained are qualitatively similar to those determined for the rearrangement of allyl vinyl ether (∼5% $^{18}O$ KIE and ∼0.9% $^{13}C$ KIE).[21,22] They indicate that the enzymatic reaction also likely proceeds through a concerted but asynchronous pericyclic transition state, with C−C bond formation lagging considerably behind C−O bond cleavage.[23] The very large $^{18}O$-effect rules out protonation of the substrate's ether oxygen at the transition state, although hydrogen bonding, for example with the proximal guanidinium group of Arg90,[24] cannot be excluded.[25,26]

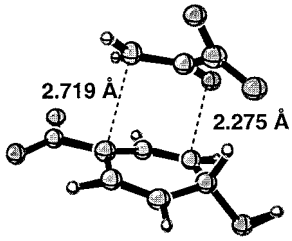


**Figure 1.** Becke3LYP/6-31G* transition structure.

Heavy atom isotope effects for the uncatalyzed mutase reaction are not yet available, but theoretical isotope effects based on the transition structure (Figure 1) obtained at the Becke3LYP/6-31G* level of theory are provided in Table 1 for the sake of comparison. Becke3LYP/6-31G* calculations have previously yielded isotope effects for a number of reactions,[27] including Claisen rearrangements,[28] that are in excellent agreement with experiment. In the present instance, the experimental $^{18}O$ isotope effect for the C75S variant is significantly larger than the predicted value, whereas the $^{13}C$ effect is smaller.[29] These differences, if confirmed experimentally, would indicate a more dissociative transition state for the enzymatic reaction than for its solution counterpart. Such a species could be stabilized electrostatically by the complementarily charged residues that line the enzyme active site.[24,30]

Our results provide valuable new information on an intensively investigated enzymatic transformation. Utilization of other chorismate derivatives, labeled for example in the enol pyruvate side chain, will allow further refinement of the structure of the enzymatic transition state, while extension of these studies to the uncatalyzed reaction and to other chorismate mutases will facilitate analysis of the factors responsible for efficient enzymatic catalysis.

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**Supporting Information Available:** Coordinates and computational details for calculation of the theoretical isotope effects (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(18) $R_0$ was determined by running the rearrangement reaction to completion (3 h) with wild-type BsCM at 2 mM chorismate and determining the isotope ratio on the mass spectrometer (experiment done in triplicate). The fractional conversion $f$ was determined by measuring the UV absorbance of the chorismate chromophore at 275 nm of the reaction mixture at the endpoint.

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