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Experimental Evidence for Enzyme-Enhanced Coupled Motion/Quantum Mechanical Hydrogen Tunneling by Ketosteroid Isomerase

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Abstract: Although there are considerable data demonstrating that quantum mechanical hydrogen tunneling (HT) occurs in both enzymatic and nonenzymatic systems, little data exist that address the question of whether enzymes enhance the amount of HT relative to the corresponding nonenzymatic reactions. To investigate whether 3-oxo-Δ^5-steroid isomerase (ketosteroid isomerase, KSI) enhances HT relative to the nonenzymatic (acetate-catalyzed) isomerization of Δ^5-androstene-3,17-dione (**1**) to Δ^4-androstene-3,17-dione (**3**), α-secondary deuterium kinetic isotope effects (KIE) at C-6 of the steroid were determined for both the KSI- and acetate-catalyzed isomerizations. The normal intrinsic secondary KIE for both wild type (WT) KSI (1.073 ± 0.023) and acetate (1.031 ± 0.010) suggest the possibility of coupled motion (CM)/HT in both the enzymatic and nonenzymatic systems. To assess the contribution of CM/HT in these reactions, the secondary KIE were also measured under conditions in which deuterium instead of hydrogen is transferred. The decrease in secondary KIE for WT (1.035 ± 0.011) indicates the presence of CM/HT in the enzymatic reaction, whereas the acetate reaction shows no change in secondary KIE for deuterium transfer (1.030 ± 0.009) and therefore no evidence for CM/HT. On the basis of these experiments, we propose that KSI enhances the CM/HT contribution to the rate acceleration over the solution reaction. Active site mutants of KSI (Y14F and D99A) yield secondary KIEs similar to that of WT, indicating that mutations at the hydrogen-bonding residues do not significantly decrease the contribution of CM/HT to the KSI reaction.

Introduction

Although transition state (TS) stabilization is generally accepted as the main source of the extraordinary rate accelerations (up to 10^{17}-fold)[1] of enzymatic reactions, the details of this stabilization have been the subject of much debate. Several mechanisms have been proposed, including low-barrier hydrogen bonds,[2,3] preorganization effects,[4,5] near attack conformations,[6] and protein dynamics effects.[7] Several authors have proposed that enzymes might accelerate hydrogen-transfer reactions by enhancing quantum mechanical tunneling relative to solution, either by inducing tunneling where it did not exist in the solution reaction or by increasing the amount of tunneling already present.[8–10] In contrast, however, theoretical considerations have led to the conclusion that tunneling effects are generally similar in enzymatic and nonenzymatic reactions.[11]

Although hydrogen tunneling (HT) is well-documented, both in solution and in enzymatic reactions,[12,13] there is little experimental evidence that demonstrates enhanced HT in an enzymatic reaction relative to the corresponding model reaction in solution. Alston et al.[8,14] found a secondary tritium kinetic isotope effect (KIE) larger than the equilibrium isotope effect (EIE) for proton transfer by triosephosphate isomerase, in contrast to the hydroxide-catalyzed enolization of acetophenone, which exhibits a KIE smaller than the EIE. They interpreted these results in terms of an enhanced tunneling contribution to the enzymatic reaction. Similarly, the secondary KIEs for yeast aldolase[15] and enoyl-CoA-hydratase[16] are also larger than the EIEs, again leading to the possibility of enhanced tunneling in these enzymatic systems. However, Doll et al. reported that the extent of tunneling for the nonenzymatic abstraction of a

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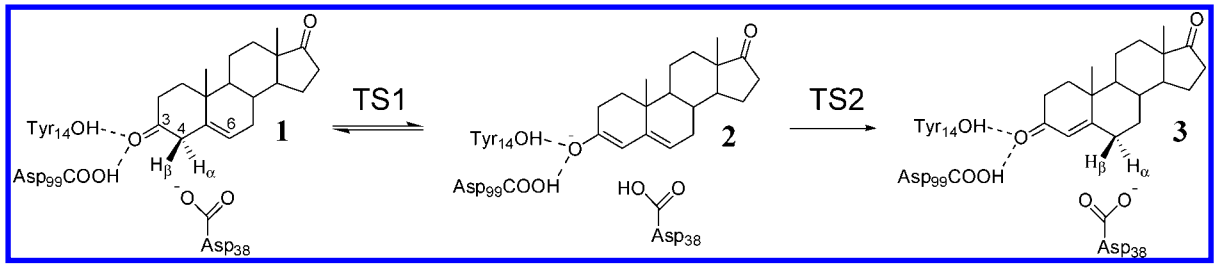
Scheme 1



hydrogen atom from ethylene glycol by coenzyme B_{12} (5′-deoxyadenosylcobalamin, AdoCbl)[17] is identical within error to that of a very similar enzymatic reaction (hydrogen atom abstraction from methylmalonyl-CoA by AdoCbl-dependent methylmalonyl-CoA mutase),[18] leading to the conclusion that there is no enhanced quantum mechanical tunneling for this enzyme. Valley and Fitzpatrick[19] found no evidence for tunneling in either the nonenzymatic (acetate or phosphate) or enzymatic (nitroalkane oxidase) deprotonation of nitroethane, again providing no basis for enhanced tunneling in an enzymatic reaction. Thus, there are only a few examples of studies that either support or refute the hypothesis that enzymes enhance quantum mechanical HT. The scarcity of experimental evidence is due to the difficulty of designing a suitable system for comparison. Not only must one find a reaction that occurs by identical mechanisms both in solution and at the active site of the enzyme, but the details of the rate-limiting step(s) must be known, and both systems must be amenable to the measurement of precise KIEs.

The ketosteroid isomerase (KSI)-catalyzed isomerization of 5-androstene-3,17-dione (**1**) to 4-androstene-3,17-dione (**3**) provides just such a system, because both the enzymatic and solution reactions have been extensively characterized,[20–24] and both the enzymatic and acetate-catalyzed reactions occur through the same mechanism (Scheme 1). KSI (3-oxo-Δ^5-steroid isomerase, EC 5.3.3.1.) uses a single active site base (Asp-38, pK_a 4.57)[25] to transfer a proton from C-4 to C-6 of the steroid, and this base can be mimicked by acetate (pK_a 4.75) in the corresponding nonenzymatic reaction. In both reactions, proton abstraction from C-4 (enolization) generates a dienolate intermediate (**2**), which is then reprotonated at C-6 (ketonization) to form the conjugated product (**3**). KSI stabilizes the intermediate and the flanking TSs (TS1 and TS2) through hydrogen bonding from Tyr-14 and Asp-99 to O-3 of the steroid.[26,27] In addition to the mechanistic information regarding these reactions, free energy diagrams and the associated rate constants for all steps have been determined for both the KSI-[28] and the acetate-catalyzed[23] reactions (Figure 1), enabling intrinsic KIEs to be calculated from the observed values.

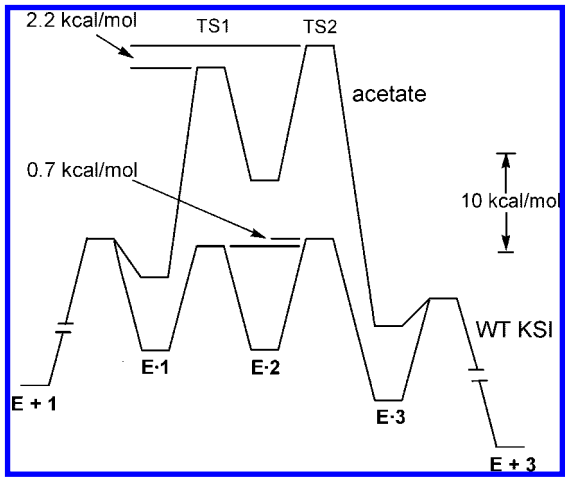


Figure 1. Free energy diagram for the isomerization of **1** to **3** catalyzed by acetate and by wild type (WT) KSI at 25 °C.

To probe for the existence of tunneling in the ketonization step, we determined secondary deuterium KIEs at C-6 for the second step of the reaction (protonation at C-6) for both enzymatic and nonenzymatic catalysis. This method is based upon the study of Huskey and Schowen,[9] in which they showed that quantum mechanical HT can result in inflated secondary hydrogen isotope effects for systems in which the isotopic hydrogen is coupled to the transferring hydrogen. In this work, we describe results of our investigation that provide evidence for enzymatic enhancement of coupled motion (CM)/HT by KSI.

Materials and Methods

Materials. WT and mutant isomerases (Y14F and D99A) were available from previous work.[29] Substrate (5-androstene-3,17-dione) was prepared as described previously.[30] All chemicals used were reagent grade or better. UV data were obtained on either a Cary 1 Bio or Cary 100 Bio spectrophotometer. IR spectra were obtained on a ThermoNicolet Avatar 320 FTIR instrument equipped with an attenuated total reflectance crystal for solid-sample analysis. NMR spectra were acquired on either a Bruker 300 MHz or Bruker Avance 500 MHz instrument. Chemical shifts are given in ppm.

5-Androstene, 6-deutero-3,17-dione (1-6D). Compound **1-6D** was prepared in seven steps, as shown in Scheme 2. This synthetic route is based on the work of Palmer et al.[31] in the preparation of C-6 tritiated cholesterol derivatives.

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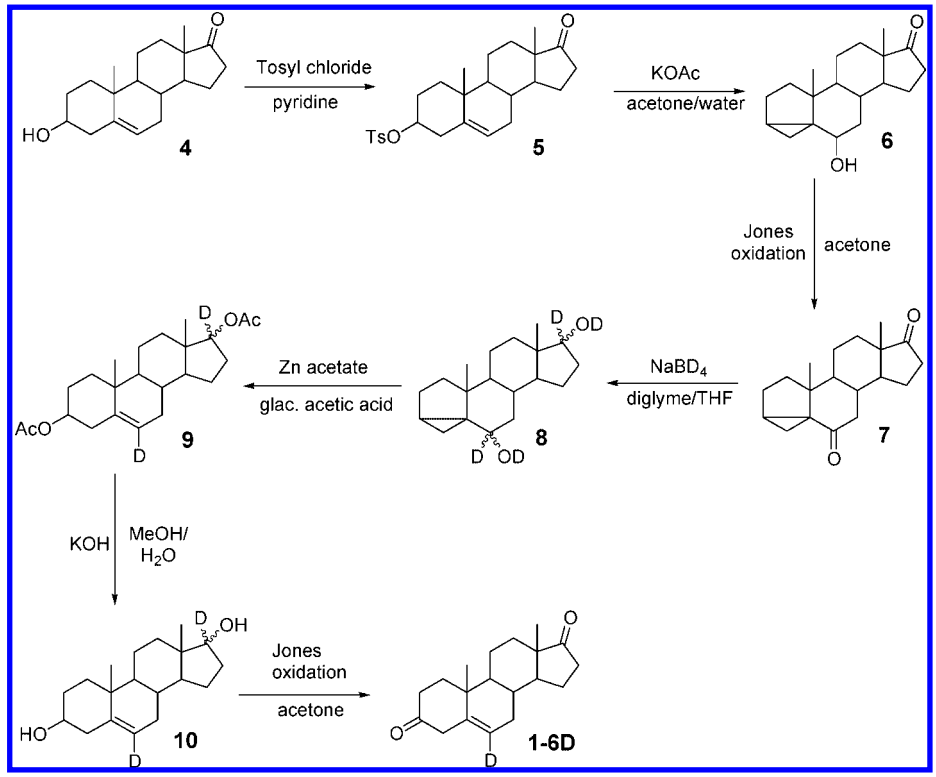
Scheme 2



3β-Tosyloxy-5-androstene-17-one (5). [32] A total of 2.884 g (10 mmol) of 3β-hydroxy-5-androstene-17-one (**4**) and 2.856 g (15 mmol) of tosyl chloride were stirred in 40 mL of dry pyridine for 20 h at room temperature. Pyridine was removed by rotary evaporation under reduced pressure, and the remaining substance was dissolved in ethyl acetate. The organic phase was washed with 1 N HCl, water, sodium bicarbonate, and water. After drying ($MgSO_4$) and filtration, the solvent was removed, and the residue was purified by column chromatography by using silica gel (Merck 60) and hexane:ethyl acetate (1:1). Recrystallization from ethyl acetate−hexane gave 85% yield (3.757 g); mp 150−152 °C (lit. mp 153−155 °C);[33] R_f 0.81 (hexane/ethyl acetate 1:1). Anal. Calcd for $C_{26}H_{34}SO_4$ (442.61): (C, 70.55; H, 7.74; S, 7.24; O, 14.45). Found: (C, 70.48; H, 7.69; S, 7.02). IR, λ_{max} (solid): 2935, 2896, 1734, 1362, 1174 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 0.86 (s, 3H, C-18 methyl), 0.99 (s, 3H, C-19 methyl), 2.44 (s, 3H, tosyl methyl), 4.35 (m, 1H, C-4 H), 5.38 (m, 1H, C-6H), 7.34 (d, 2H, J = 8 Hz), 7.79 (d, 2H, J = 8 Hz)ν.

3,5 Cyclo-6-hydroxy-androstane-17-one (6). Compound **5** (3.642 g, 8.23 mmole) and potassium acetate (4.03 g, 41 mmol) in an acetone/water (80 mL:20 mL) mixture were refluxed for 18 h. The acetone was removed by rotary evaporation, and the residue was extracted with ethyl acetate, washed with water, and dried ($MgSO_4$). Purification, as described for compound **5**, gave 85% yield (2.02 g); mp 128−130 °C (lit. mp 132−135 °C);[34] R_f 0.60 (hexane/ethyl acetate 1:1). Two spots were apparent by thin-layer chromatography (TLC), which are likely the two (α and β) isomers of the C-6 alcohol group. Anal. Calcd for $C_{19}H_{28}O_2$ (288.43): (C, 79.12; H, 9.78; O, 11.09). Found: (C, 78.77; H, 9.81; O, 11.05). IR, λ_{max} (solid): 3596, 3060, 2997, 2861, 1720 cm^{-1}.

3,5 Cyclo-androstane-6,17-dione (7). A total of 5 mL of Jones reagent was slowly added to a solution of compound **6** (1.9 g, 6.59 mmol) in 20 mL of acetone (previously cooled to ~5 °C). The mixture was stirred at this temperature for 20 min, at which point excess Jones reagent was destroyed by addition of methanol. Water was added, and the product was extracted with ethyl acetate, washed with water, and dried ($MgSO_4$). Ethyl acetate was removed, and the product was purified by column chromatography by using silica gel (Merck 60) and hexane:ethyl acetate (1:1). Recrystallization from methanol gave 63% yield (1.18 g); mp 188−190 °C; R_f 0.65 (hexane/ethyl acetate 1:1). Anal. Calcd for $C_{19}H_{26}O_2$ (286.41): (C, 79.67; H, 9.15; O, 11.17). Found: (C, 79.33; H, 9.18; O, 11.14). IR, λ_{max} (solid): 2956, 1731, 1679 cm^{-1}.

3,5 Cyclo-6,17-D-androstane-6,17-diol (8). Compound **7** (1.00 g, 3.49 mmol) of and 467 mg (11.15 mmol) of sodium borodeuteride ($NaBD_4$, >99 atom % D) were dissolved in 30 mL of diglyme:THF (1:1) and heated for 24 h. Solvents were removed by rotary evaporation under reduced pressure. Water and ethyl acetate were added, and the product was extracted into the ethyl acetate fraction and dried ($MgSO_4$). After filtration and removal of the solvent, the residue was purified by column chromatography by using silica gel (Merck 60) and hexane:ethyl acetate (4:1). Recrystallization from ethyl acetate gave 88% yield (905 mg); mp 181−183 °C. Anal. Calcd for $C_{19}H_{28}D_2O_2$ (292.45): (C, 78.03; D + H, 10.34; O, 10.94). Found: (C, 77.40; H, 10.31; O, 11.22). IR,

(32) We later found this synthetic method to be irreproducible, and we have since developed an easier, more reproducible method of tosylation based on the work of Velusamy et al. (Velusamy, S. Kirankunar, J. S. Punniyamurthy, T. *Tetrahedron Lett.* **2004**, *45*, 203) In a flask equipped with a Dean-Stark trap, 2.884 g (10 mmol) of 3-β-hydroxy-5-androstene-17-one, 1.90 g (10 mmol) of *p*-toluenesulfonic acid monohydrate and 119 mg (5 mol%) of cobalt hexahydrate in 40 mL of 1,2-dichloroethane were stirred and refluxed for 45 minutes. Solvent was removed under reduced pressure, and the residue was dissolved in ethyl acetate. The organic phase was washed in 1 N HCl, water, sodium bicarbonate, and water. After drying ($MgSO_4$) and filtration, the solvent was removed, and the residue was purified by column chromatography by using silica gel (Merck 60) and hexane:ethyl acetate (1:1). Recrystallization from ethyl acetate−hexane gave 83% yield (3.667 g).

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λ_{max} (solid): 3396, 3324, 2949, 2868, 2160, 1040 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): δ 0.27 (t, 1H), 0.61 (m, 1H), 0.77 (s, 3H), 0.93 (s, 3H).

5-Androstene-6-D-3,17-diol-acetate (9). Compound **8** (855 mg, 2.92 mmol) and zinc acetate (2.137 g, 11.68 mmol) were dissolved in 30 mL of glacial acetic acid and refluxed for 20 h. Solvent was removed, and the product was extracted and purified as described for compound **8**. Recrystallization from methanol gave 87% yield (958 mg); mp 160−161 °C (lit. mp (undeuterated) 158−159.5 °C);[35] R_f 0.68 (hexane/ethyl acetate 4:1). Anal. Calcd for $C_{23}H_{32}D_2O_2$ (376.53): (C, 73.36; H + D, 9.10; O, 16.99). Found: (C, 73.29; H, 9.07; O, 17.53). IR, λ_{max} (solid): 2948, 2877, 2210, 1730, 1246, 1040 cm^{-1} (lit. IR λ_{max} (KBr): 1730, 1620, 1250, 1040 cm^{-1}).[35]

5-Androstene-6-D-3,17-diol (10). Compound **9** (871 mg) was added to a solution of 500 mg of potassium hydroxide in 25 mL of methanol/water (4:1), and the solution was refluxed for 3 h. After cooling, the precipitated solid was filtered off, washed with water until the filtrate pH was neutral, dried, and recrystallized from ethyl acetate to give 66% yield (443 mg); mp 176−179 °C (lit. mp 178−179).[35] Anal. Calcd for $C_{19}H_{28}D_2O_2 \cdot H_2O$ (310.47): (C, 73.50; H + D, 10.39; O, 15.46). Found: (C, 73.35; H, 10.35; O, 15.09). IR, λ_{max} (solid): 3466, 3199, 2941, 2881, 2826, 2227, 1654, 1434, 1374, 1056, 1032 cm^{-1} (lit. IR λ_{max} (KBr): 3400, 1620, 1050 cm^{-1}).[35]

5-Androstene-6-D-3,17-dione (1-6D). A total of 1 mL of Jones reagent was added dropwise to 98.6 mg (0.34 mM) of compound **10** dissolved in 20 mL of acetone previously cooled to ~0 °C. After 3 min, excess Jones reagent was destroyed by addition of methanol. A cold, saturated NaCl solution was added to the reaction mixture, and the product was extracted with an excess of ethyl acetate. The organic phase was washed with water, dried ($MgSO_4$), and filtered. Solvent was removed by rotary evaporation under reduced pressure at room temperature. The product was purified by column chromatography by using silica gel (Merck 60) and hexane/ethyl acetate (6:4). Recrystallization from ether gave 39.5% yield (40 mg). IR, λ_{max} (solid): 2966, 2941, 2884, 2860, 2824, 2236, 1736, 1708, 1456, 1392, 1374, 1022, 1012 cm^{-1}. Mass spectrometry (MS, APCI, low resolution) 288.1 *m/z*. ^1H NMR ($CDCl_3$, 500 MHz, integration of the C-6H (5.36 ppm) peak relative to the C-4βH (3.28 ppm) and C-4αH (2.84 ppm) peaks in the 500 MHz ^1H NMR of **1-6D** shows that >99% deuteration at C-6 was achieved by this method): δ 3.28 (dt, 4β-H), 2.84 (dd, 4α-H), 1.21 (s, 19-Me), 0.92 (s, 18-Me). lit. δ 3.28 (dt, 4β-H), 2.84 (dd, 4α-H), 5.36 (dt, 6-H), 1.20 (s, 19-Me), 0.90 (s, 18-Me).[36] No signal was observed at 5.36. TLC of the product showed only one spot. R_f (hexane/ethyl acetate (3:2)) 0.66.

Autoxidation during Isomerization. Autoxidation products were identified by using LC/MS, ^1H NMR, IR, and UV spectroscopy and high-resolution MS (Michigan State University Mass Spectrometry Facility). To acquire sufficient sample for these analyses, a large-scale hydroxide-catalyzed isomerization was performed. Methanol (120 mL) containing 100 mg of **1** was added to 480 mL of 0.8 mM NaOH in a 1 L separatory funnel. The solution was mixed and left open to air. After 40 min, the UV spectrum had stopped changing, and the reaction mixture was extracted with chloroform (3 × 60 mL). The organic layers were combined, dried ($MgSO_4$), and filtered. After evaporation of the filtrate to dryness under reduced pressure, the dried extracts (114 mg) were dissolved in 4 mL of chloroform and loaded on a preparatory TLC plate (silica gel, Merck 60). The first separation by using a 60:40 hexane:ethyl acetate mobile phase did not provide adequate separation of bands; therefore, the plate was run a second time using the same solvent mixture. Individual bands were extracted by using a 60:40 $CHCl_3$:MeOH mixture, filtered, and evaporated to dryness.

The procedure above was repeated for the acetate-catalyzed formation of side products, except that a 2.0 M potassium acetate solution was used instead of dilute hydroxide. To repeat these reactions in the absence of air, a vacuum was applied to a three-neck round-bottom flask containing the reaction mixture (prior to addition of **1**), followed by addition of argon. This process was repeated twice more prior to the addition of **1**. Positive argon pressure was maintained throughout the reactions to avoid introduction of air.

LC/MS analysis was performed by using an Agilent 1100 HPLC connected to a Bruker Daltronics Esquire 3000 Plus (ion trap) mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) accessory. A 2.0 × 250 mm C_{18} column (Beckman Ultrasphere 5 μm) was used with a 0.4 mL/min flow rate. Isocratic elution by using 55:45 methanol:water mobile phase (15 min) was followed by an 80:20 methanol:water column wash with 0.5% acetic acid present throughout. Postcolumn mobile phase was injected directly into the mass spectrometer for analysis. MS parameters: nebulizer, 50.0 psi; dry gas, 4.0 L/min; dry gas temperature, 350 °C; APCI temperature, 450 °C; positive ion mode scanning, 120−800 *m/z*.

Deuteration of 1 and 1-6D at C-4. Compound **1** (1−2 mg) was incubated in 20−30 mL of 1.5 M potassium acetate buffer dissolved in D_2O (containing 20% CH_3OD and 1 mM ethylenediamine tetraacetic acid, EDTA) for about 1 h to exchange the C-4 protons for deuterium. The reaction was halted by extraction of the steroids with methylene chloride (3 × 10 mL). The extracts were combined and evaporated to dryness at reduced pressure and temperature (5−15 °C) to avoid isomerization. The remaining solids were purified by HPLC by using a Waters Symmetry C_{18} column (19 × 150 mm) on a Waters 600 with isocratic elution (70:30 methanol: water). Fractions containing 5-androstene-4,4-dideutero-3,17-dione (**1-4D2**, λ_{max} = 290 nm, retention time = 7 min) were collected and evaporated to dryness by using a vacuum pump at reduced temperature (5−15 °C).

The same procedure was used with the **1-6D** substrate to create a triply deuterated substrate, 5-androstene-4,4,6-trideutero-3,17-dione (**1-4D$_2$6D**). Purity of these C-4 deuterated substrates was assessed by HPLC, which showed only two peaks corresponding to the substrate and a small amount of product. UV analysis showed less than 2% of product. The extent of deuteration was assessed by ^1H NMR (500 MHz) in $CDCl_3$ (99.8 atom %D, 0.05% TMS, Cambridge Isotope Laboratories). Integration of the remaining ^1H NMR C-4H peaks (3.36 and 2.84 ppm) relative to the C-18 methyl peak (0.9 ppm) showed that >99% deuteration at both the C-4α and C-4β positions was achieved by this process.

KIEs in H_2O. As described by Vitullo and Logue,[37] small KIEs can be accurately determined in a noncompetitive manner by the simultaneous UV monitoring of the reactions of unsubstituted and isotopically substituted molecules. In these experiments, a UV−vis spectrophotometer with a cell changer capable of switching between six cuvettes was used to monitor six separate reactions simultaneously. KIEs in acetate buffer were determined by adding identical amounts of acetate buffer and methanol to each cuvette, followed by addition of **1** (dissolved in methanol) to three cuvettes and **1-6D** to the other three cuvettes. The UV absorbances were monitored at 248 nm for 6−10 half-lives (~20−60 h, 98.5−99.9% completion). Infinity points were observed to be stable. Final conditions in the cuvettes were 3.3% methanol, 1 mM EDTA, 1−3 M acetate buffer, and 25.0 °C. The concentrations of **1** and **1-6D** were identical to each other for each set of runs but varied between 6 and 40 μM in separate sets.

The data were edited to be evenly weighted according to changes in absorbance.[38] KIEs were calculated by averaging the first-order rate constants of the three cuvettes containing **1** and dividing this number by the average of the rate constants from the three **1-6D**

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Scheme 3

$$E + \mathbf{1} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}\mathbf{1} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E{\cdot}\mathbf{2} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E{\cdot}\mathbf{3} \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + \mathbf{3}$$

cuvettes. At least three such values were determined and averaged to obtain each reported KIE.

Enzymatic KIEs were determined in a similar manner. Six cuvettes were prepared with phosphate buffer (4 mM, pH 7.0, 1.0 mM EDTA), methanol, and substrate or deuterated substrate. Then, identical volumes of enzyme (10−20 μL, diluted in 2.5 mg/mL bovine serum albumin (BSA)) were added to all six cuvettes. Final substrate concentrations in the cuvettes were between 6 and 12 μM. Catalyst concentrations were as follows: acetate (1−3 M); WT (5−7 pM for 3.3% methanol, 195−225 pM for 20% methanol); Y14F (80−125 nM); and D99A (12−16 nM). Enzyme-catalyzed reactions were monitored at 248 nm for 90−120 min (7−10 half-lives, 99−99.9% completion). Data analysis was performed in a manner identical to that used for acetate.

KIEs in D_2O. KIEs in D_2O were determined in a manner similar to that used to determine KIEs in H_2O with the following modifications. A solution of 4 mM deuterated phosphate buffer (KPi), containing 1 mM EDTA, was used for the enzymatic runs. Acetate buffers (3−4 M, pD 7.0), containing 1 mM EDTA, were prepared by dissolving potassium acetate and $Na_2EDTA{\cdot}2H_2O$ in D_2O (99.9 atom % D) and adjusting the pD with small quantities of glacial acetic acid. The percent of ^1H for all deuterated buffers is calculated to be <0.2%. Deuterated methanol (CH_3OD) was used in place of protonated methanol, and the 2.5 mg/mL BSA solutions used for enzyme dilution were made with deuterated 4 mM KPi buffer. Substrates used were 5-androstene-4,4-dideutero-3,17-dione (**1-4D$_2$** for k_H) and 5-androstene-4,4,6-trideutero-3,17-dione (**1-4D$_2$6D** for k_D). Enzyme concentrations were increased by 5−8-fold over those used in protonated solvents to account for primary hydrogen and solvent KIEs.

Intrinsic KIE Calculation for the WT Reaction. Reactions catalyzed by acetate,[23] Y14F,[29,39] and D99A[29,39] all have clean rate-limiting ketonization steps; therefore, the observed KIEs are the intrinsic KIEs for these catalysts. However, ketonization is not cleanly rate-limiting for the WT-catalyzed reaction;[28] therefore, the observed KIEs are smaller than the intrinsic KIEs. Because the relative heights of the TSs for the individual kinetic steps are known for the solvent systems used, the intrinsic KIEs can be calculated from the observed KIEs by using eq 1[40]

$$V_D/K = (k_D + C_f + C_r \times \mathrm{EIE})/(1 + C_f + C_r) \qquad (1)$$

in which V_D/K is the observed KIE on k_{cat}/K_m, k_D is the intrinsic KIE, C_f and C_r are the forward and reverse commitment factors, respectively, and EIE is the EIE between reactant and product. Because the microscopic rate constants for isomerization by WT are known,[38] C_f and C_r (and therefore the intrinsic KIE) can easily be calculated. For the mechanistic scheme shown (Scheme 3), where k_5 (ketonization) is the isotopically sensitive step, $C_f = (k_5/k_4)[(k_3 + k_2)/k_2]$ and $C_r = k_6/k_7$. Because $k_6 \ll k_7$,[28] $C_r = 0$, and it can be ignored. At high methanol concentrations (20%) $k_2 \gg k_3$, so $C_f = k_5/k_4$, whereas at low methanol concentrations, k_2 and k_3 are similar, so we must use $C_f = (k_5/k_4)[(k_3 + k_2)/k_2]$ for the intrinsic KIE calculations. It should be noted that only the protonation of the intermediate at C-6 is stereospecific.[41]

KIE of D99A by MS. A known mixture of undeuterated (**1**) and C-6 deuterated (**1-6D**) compounds was isomerized by D99A in dilute phosphate buffer in the following manner. A solution of 10 mL of methanol containing a ~2:1 mixture of **1**:**1-6D** was added to 290 mL of 6 mM KPi buffer (pH 7.0, 1 mM EDTA). A 40 mL aliquot of this pre-enzyme solution was extracted with methylene chloride (3 × 10 mL). Solvent was removed under reduced pressure, and the solids were redissolved in methanol. This solution was labeled 0% reaction and stored at −80 °C to await HPLC separation of substrate and product. A solution of 200 μM D99A (8 μL) was added to the remaining 260 mL of pre-enzyme solution. Final conditions were 3.3% methanol, [**1**] = 11.2 μM, [**1-6D**] = 4.5 μM, and [D99A] = 6.2 nM. Aliquots were taken at 10, 50, and 85% reaction (determined by UV absorbance at 248 nm) and worked up identically to the pre-enzyme aliquot. Finally, 1 μL of WT (336 μM) was added to the residual solution to drive the reaction to completion, and the remaining solution was worked up and labeled 100% reaction.

The five time point extracts (0, 10, 50, 85, and 100% reaction) were separated by HPLC as described earlier. The substrate and product fractions were collected and evaporated to dryness under high-vacuum at 5−15 °C. These fractions were then dissolved in methanol and analyzed by electrospray mass spectrometry (ESI-MS). Individual peaks from resulting mass spectra were integrated by summing the observed intensities in 0.025 *m/z* increments over the entire peak (e.g., for the 287 *m/z* peak, the intensities between 286.6 and 287.4 were summed). Peak integrations obtained were used to determine C-6H(D) KIEs.

The ratio of the M + 1 and M + 2 peaks (287 and 288 *m/z*, respectively) was used to determine the ratio of deuterated/nondeuterated substrate and product at each time point (see Supporting Information). Equations 2 and 3 use these ratios to determine the isotope effect based on substrate and product ratios, respectively,[42,43]

$$\mathrm{KIE} = k_H/k_D = \log(1 - F)/\log[(1 - F)R_{SF}/R_0] \qquad (2)$$

$$\mathrm{KIE} = k_H/k_D = \log(1-F)/\log[(1 - F)R_{PF}/R_0] \qquad (3)$$

where F is the fraction of reaction completed, R_{SF} is the ratio of [**1-6D**]/[**1**] at fraction F, R_{PF} is the ratio [**3-6D**]/[**3**] at fraction F, and R_0 is the [**1-6D**]/[**1**] ratio at the start (or [**3-6D**]/[**3**] at the end) of the reaction.

Direct Injection ESI-MS. Mass spectra of the substrate and product were acquired by dissolving the steroid in methanol (100 μM) and injecting the solution directly into the mass spectrometer (Bruker Daltronics Esquire 3000 Plus). A total of 50 μL/min of steroid solution was mixed with 500 μL/min of 60:40 methanol: 0.5% acetic acid solution by using a T-connector. Mass spectrometer parameters were as follows: total injection flow rate, 550 μL/min; APCI temperature, 450 °C; dry gas (N_2) temperature, 350 °C; dry gas flow rate, 5.0 L/min; and selective ion monitoring between 282.5 and 292.5 *m/z*. Spectra were acquired for 6−10 min and averaged.

Results

Autoxidation during Base-Catalyzed Isomerization. Initial attempts to measure the KIE of the acetate-catalyzed isomerization in the absence of EDTA indicated that the reaction does not proceed cleanly. Final UV absorbances were 10−15% lower than calculated, and the kinetic data did not fit well to a first-order equation. For these reasons, product analysis was undertaken.

(38) The data were manually edited such that they were evenly weighted according to changes in absorbance. Typically, five absorbance readings per 0.001 AU increase were kept, and the rest of the data was discarded.
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(42) Strictly speaking, these equations are appropriate for analyses when the isotopic molecule is present in trace amounts. Because of the small KIEs measured here, the errors introduced by using these simpler equations are less than 0.5%, which is considerably smaller than the experimental error.
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Scheme 4

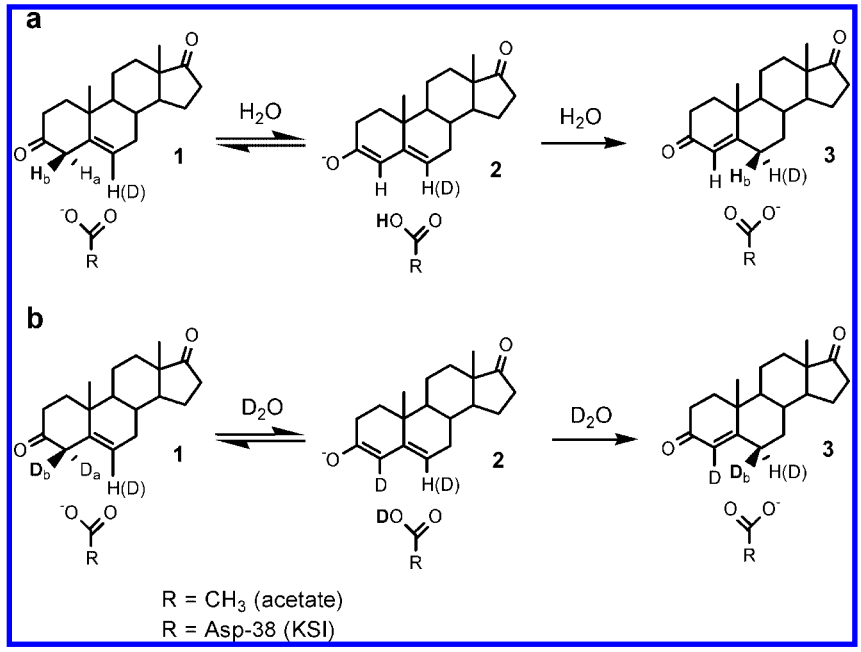

Table 1. Rate Constants ($M^{-1}s^{-1}$) for the Acetate- and KSI-Catalyzed (k_{cat}/K_m) Isomerizations of **1** at 25 °C

	H transfer		D transfer	
catalyst[a]	**1**	**1-6D**	**1-4D$_2$**	**1-4D$_2$6D**
acetate	$(28.1 \pm 1.1) \times 10^{-6}$ [c]	$(27.2 \pm 1.4) \times 10^{-6}$	$(4.6 \pm 0.3) \times 10^{-6}$	$(4.4 \pm 0.3) \times 10^{-6}$
WT	$(13.7 \pm 0.8) \times 10^{7}$ [d]	$(13.3 \pm 0.8) \times 10^{7}$ [e]	$(4.07 \pm 0.05) \times 10^{7}$	$(3.99 \pm 0.04) \times 10^{7}$
WT[b]	$(45.7 \pm 1.9) \times 10^{5}$	$(43.3 \pm 1.4) \times 10^{5}$	$(8.2 \pm 0.8) \times 10^{5}$	$(8.0 \pm 0.8) \times 10^{5}$
Y14F	7320 ± 100[f]	6910 ± 60[g]	890 ± 10	860 ± 10
D99A	$(75 \pm 8) \times 10^{3}$	$(70 \pm 8) \times 10^{3}$	$(7.53 \pm 0.04) \times 10^{3}$	$(7.25 \pm 0.06) \times 10^{3}$

[a] 3.3% methanol was used, unless otherwise noted. [b] 20% methanol. [c] Literature values (Lit.) 30.9×10^{-6} [21] and 32×10^{-6} $M^{-1}s^{-1}$.[23] [d] Lit. 24×10^{7} $M^{-1}s^{-1}$[25] and 19.8×10^{7} $M^{-1}s^{-1}$.[36] [e] Lit. 19.8×10^{7} $M^{-1}s^{-1}$.[36] [f] Lit. 8690 $M^{-1}s^{-1}$.[48] [g] Lit. 8520 $M^{-1}s^{-1}$.[48]

The products of the acetate-, hydroxide-, and WT-catalyzed isomerizations of **1** in the absence of EDTA were analyzed by LC/MS. The WT reaction shows essentially complete isomerization of **1** to **3**, with negligible side-product formation. The acetate and hydroxide reactions, on the other hand, occur with significant side-product formation, as evidenced by additional peaks in the chromatogram. The most prominent side product was identified as 4-androstene-3,6,17-trione (**11**), based on its λ_{max} (250 nm) and M + 1 of 301 *m/z*. This identification was confirmed by a comparison of the HPLC retention time, ^{1}H NMR, IR, UV, and mass spectra of the isolated product with those for authentic **11** (Steraloids), as well as with literature values.[44,45] Finally, the high resolution mass spectrum of the isolated product (M + 1 *m/z* = 301.1805) is consistent with the molecular formula ($C_{19}H_{24}O_3$) of **11**.

The ^{1}H NMR spectrum of the acetate-catalyzed isomerization product mixture exhibits two major peaks in the vinyl proton region (4−7 ppm), corresponding to the isomerization product **3** (5.75 ppm) and the side product **11** (6.22 ppm). Integration of these peaks gave a ratio of about 3:1 favoring **3**, whereas an identical reaction under argon gave a ratio of 7:1. For the hydroxide-catalyzed reactions, a similar analysis yielded 3:1 and 40:1 ratios, respectively. Although attempts to eliminate oxygen did reduce formation of **11**, a simpler and more efficient method is the addition of EDTA to the buffer solution. Addition of 1 mM EDTA was shown to eliminate side-product formation (HPLC).[21]

C-6H(D) α-Secondary KIEs. The α-secondary deuterium KIEs at position C-6 of the substrate were obtained from the pseudofirst-order rate constants in the presence of EDTA for the acetate- and KSI-catalyzed isomerizations under nonsaturating conditions ([S] ≪ K_M) by using UV spectroscopy. Reactions were monitored to greater than ≥98% completion, and both acetate- and enzyme-catalyzed reactions showed excellent pseudofirst-order kinetics. These KIEs were determined for both hydrogen and deuterium transfer in H_2O and D_2O, according to the reactions shown in Scheme 4 panels a and b, respectively. The second-order rate constants of isomerization for the different catalysts and conditions are given in Table 1.

In order to appropriately analyze these data, it is necessary to calculate the intrinsic isotope effect for TS2 from the observed rate constants. This intrinsic isotope effect for WT was calculated from eq 1, as described in the Materials and Methods, by using the method described by Northrop.[46] This model corrects the observed isotope effect to account for the extent to which the isotope-dependent step is rate-limiting. For the mutants and acetate, the intrinsic effect is equal to the observed effect because the rate-determining step is cleanly ketonization. Intrinsic α-secondary KIEs for ketonization of the intermediate are given in Table 2. To determine whether the secondary KIEs determined in protonated and deuterated buffers are different, the Student t test[47] was applied to the intrinsic KIEs for the enzymatic data.

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Table 2. Observed and Intrinsic C-6 α-Secondary Deuterium KIEs for the Isomerization of **1** by Various Catalysts (UV Spectroscopy) at 25 °C

	H transfer[a]		D transfer[g]		
catalyst[b]	KIE_{obsd}	KIE_{intr}	KIE_{obsd}	KIE_{intr}	Student t test[d]
acetate	1.031 ± 0.013	1.031 ± 0.013	1.030 ± 0.009	1.030 ± 0.009	ND
WT	1.033 ± 0.011	1.066 ± 0.023[e]	1.021 ± 0.005	1.029 ± 0.007[f]	>98%
WT[c]	1.055 ± 0.017	1.073 ± 0.023[g]	1.029 ± 0.009	1.035 ± 0.011[h]	>99.5%
Y14F	1.059 ± 0.014	1.059 ± 0.014	1.038 ± 0.008	1.038 ± 0.008	>90%
D99A	1.075 ± 0.004	1.075 ± 0.004	1.038 ± 0.005	1.038 ± 0.005	>99.9%

[a] Error values represent one standard deviation. [b] 3.3% methanol was used, unless otherwise noted. [c] 20% methanol. [d] Probablility that the intrinsic KIEs for H and D transfers are different. ND = not determined. [e] $C_f = 0.99$. [f] $C_f = 0.29$. [g] $C_f = 0.33$. [h] $C_f = 0.21$.

KIE by MS. The C-6H(D) KIE for the D99A-catalyzed isomerization in protonated buffer was also determined by MS. In these experiments, a ~2:1 mixture of **1** and **1-6D** was isomerized by D99A, and portions of this solution were quenched at different time points during the reaction. At each time point, substrate and product were separated by HPLC, collected and individually analyzed by direct injection MS. KIEs were determined by using the 287/288 *m/z* ratios at three reaction times, as described in Materials and Methods (KIE in parentheses): 10% reaction (1.04), 50% reaction (1.08), and 85% reaction (1.05, 1.10), giving an average value of 1.07 ± 0.03.

Discussion

Autoxidation during Isomerization of 1. In order to accurately determine KIE, it is imperative that the reactions lead cleanly to only one product. Oxidation during the isomerization of **1** is a potential complicating reaction that must be considered. Autoxidation of unsaturated 3-keto steroids was first described in 1961 by Camerino et al., who observed the base-catalyzed oxidation of α,β-unsaturated systems at C-4 and C-6.[49] Ringold and Malhotra subsequently detected the uncatalyzed autoxidation and isomerization of purified β,γ-unsaturated 3-keto steroids upon standing at room temperature.[50] Finally, de la Mare et al. observed the acid-catalyzed autoxidation of β,γ-unsaturated 3-keto steroids to form a number of C-6 oxidized products.[51] Dienol(ate)s are thought to be the intermediates in all of these reactions.[49,51,52]

We observed large quantities (15−25%) of the oxidation product 4-androstene-3,6,17-trione (**11**), as well as other side products, during both the acetate- and hydroxide-catalyzed isomerizations of **1**. Support for oxygen as a reactant in this process is provided by the reduction in side-product formation when **1** is incubated with hydroxide under argon. The fact that oxidation side products are not completely eliminated is likely due to incomplete oxygen removal, as observed previously.[50] A more effective method of avoiding oxidative side-product formation, which was employed by de la Mare et al.,[51] involves addition of the metal-chelating agent EDTA. We find 1 mM EDTA to be sufficient to reduce formation of **11** to <1% of the reaction products, giving clean conversion of **1** to **3** and allowing the observation of first-order isomerization kinetics from which KIEs can be determined.

Reliability of the Isotope Effects. Because the α-secondary KIEs were determined noncompetitively by simultaneous measurements of the pseudofirst-order isomerization rates of protonated and deuterated substrates, it is necessary to demonstrate that this method produces reliable isotope effects. Although this technique has been reported to be effective for determining KIEs to 1% accuracy,[37] the use of separate cuvettes introduces the possibility that the observed KIEs are due to slight differences in the solutions or the conditions rather than to actual isotope effects. One concern is temperature control of the cuvettes. To check for warm/cool spots, six cuvettes containing identical amounts of buffer (4 mM KPi, pH 7.0), methanol (20%), substrate (5.3 μM), and WT (167 pM) were prepared, and the reaction was monitored for 6−10 half-lives for each cuvette. This experiment was repeated six times, and differences between rate constants determined in any two wells were <1%, demonstrating that temperature variations among solutions are insignificant. As an added precaution, the cuvette wells containing protonated and deuterated substrates were switched from run to run, such that any small differences in temperature between wells would average out. For noncompetitive determinations of KIEs, the purity of the reactants is an important consideration, which was addressed by a two-step purification process. The substrates were first purified by column chromatography and then further purified by reverse-phase HPLC. LC/MS analysis showed no detectable impurities for both **1** and **1-6D**.

In addition, both the direction (inverse versus normal) and magnitude of the isotope effects were determined by a competitive method for D99A. Mass spectral measurement of the KIE by ESI-MS, by using a mixture of **1** and **1-6D**, confirmed that the KIE for the D99A reaction is normal and small (1.07 ± 0.03), in agreement with the kinetic results by UV (1.059 ± 0.014), validating the noncompetitive UV data. Because of the larger errors of the mass spectrometric method, only data from the UV method were used to calculate the reported KIEs.

Because of the slow rate of the acetate-catalyzed isomerization ($k \approx 3 \times 10^{-5}$ s^{-1} at the lowest acetate concentration), contributions from the uncatalyzed isomerization reaction must be considered for this system. By using data recently obtained[21] in acetate buffer (pH 5.5), the uncatalyzed rate constant is calculated to be ~1×10^{-6} s^{-1}, or 3% of the slowest acetate rate. The observed KIE could therefore contain a small[53] component due to a KIE on the uncatalyzed reaction. However, an increase in the acetate concentration by 3-fold (thereby decreasing the uncatalyzed contribution to 1%) does not change the observed KIE, indicating that the contribution of the uncatalyzed reaction to the observed KIE is small. Because

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(53) By assuming that the KIE of the uncatalyzed reaction is within 15% of the observed KIE, the contribution from the uncatalyzed rate is less than 0.5%.

the enzymatic rates are ca. 1000-fold faster than the buffer rate, buffer contribution to the observed enzymatic KIEs is negligible.

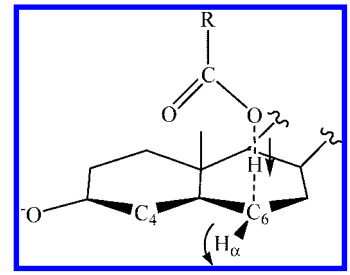
Finally, it should be noted that this work provides a much more precise knowledge of the k_H / k_D ratios (Table 2) than of the k_H and k_D values themselves (Table 1). Although reproducibility of the observed KIEs is generally 0.5−1.5%, the variation in individual rate constants is typically 4−5%. A similar result was observed previously by Vitullo et al.[37] and is likely due to slight differences in temperature and solution from run to run or day to day. Based upon the above analysis, we are confident that the KIEs in Table 2 are accurate, although they are somewhat higher than the values of 1.00 ± 0.01[36] and 1.02 ± 0.02[48] for WT and Y14F, respectively, previously reported by Xue et al. by using initial rate kinetics, a method that is more prone to error.

(Ab)normal α-Secondary KIEs. In this work, we have isolated the intrinsic secondary KIE for the protonation of the intermediate with a deuterium at C-6 (Scheme 1, TS2) by correcting the observed effects to account for the extent to which the isotope-dependent step is rate-limiting. For both the enzymatic (WT) and nonenzymatic reactions, the protonation step (TS2) is stereospecific, with protonation occurring at the β-face of the steroid.[41] Thus, the TSs for these reactions are comparable.

Secondary hydrogen isotope effects are often used to estimate the structure of TSs of both solution[43,54] and enzymatic reactions.[55–57] These secondary isotope effects normally lie between unity and the EIE. Isotope effects near unity indicate a substrate-like TS, and isotope effects approaching the EIE suggest a product-like TS. Hybridization changes are generally considered to dominate isotope effects for a hydrogen attached to a carbon undergoing a change in hybridization.[58] Both calculations and experimental evidence give α-secondary EIEs for deuterium bound to a carbon changing from sp^2 to sp^3 hybridization of about 0.89.[56,58–60] On the basis of hybridization changes, C-6 deuterium KIEs between 1.00 and 0.89 are expected for the isomerization of **1-6D**(TS2), where a KIE close to 1.00 would indicate an intermediate (**2**)-like TS, and a KIE closer to 0.89 would suggest a product (**3**)-like TS. In contrast to this expectation, however, the observed values for all of the systems investigated are greater than unity, inconsistent with a model based on hybridization changes. These values, then, must be interpreted in terms of a different model.

CM/HT. Inflated isotope effects in hydrogen-transfer reactions, such as those observed here, are diagnostic of CM or CM combined with HT.[9,61,62] CM occurs when one of the vibrations of the α-hydrogen is part of the reaction coordinate (Scheme 5), leading to an increase in the KIE as part of the α-hydrogen's vibrational mode is converted to a translation.[62]

Scheme 5



In this case, the C−H bending mode is partially lost at the TS, leading to an inflated isotope effect. By using this model, CM between the transferred proton (from Asp-38 or acetic acid) and the α-hydrogen at C-6 could account for an isotope effect larger than that expected from an analysis of hybridization changes. Computational studies[9,61,63] modeling hydrogen transfers to and from carbon indicate that semiclassical CM (without HT) can lead to secondary KIE inflations on the order of 3−4%. Therefore, CM without HT could account for part, but not all, of the KIEs observed in Table 2 (1.031−1.075, H transfer). However, much larger increases of secondary KIEs (up to 25%) can be observed when CM is combined with tunneling of the transferred hydrogen,[9,62,63] suggesting that these reactions may include a tunneling component.

One probe for the existence of HT makes use of the fact that a particle's ability to tunnel is inversely related to its mass. Thus, replacement of the transferring proton with a deuteron decreases the contribution of tunneling to the reaction, causing a smaller inflation in the secondary KIE[62] if CM/HT is significant. This method has been used to provide evidence for CM/HT in the conversion of formate to carbon dioxide by yeast formate dehydrogenase. This reaction has an extremely inflated secondary KIE (1.22) relative to the EIE (0.89).[64] When deuterium is transferred, however, the secondary KIE drops to 1.07, indicating the existence of CM/HT in this system. Similarly, the reaction of glutamase mutase with [5′-^{3}H]-adenosylcobalamin and L-glutamate shows a reduced secondary KIE when deuterium is abstracted from glutamate, which was interpreted in terms of CM/HT.[65] In contrast, the reaction catalyzed by enoyl-CoA hydratase (crotonase), which also exhibits an inflated KIE,[16] shows no change in the secondary KIE upon replacement of the transferred hydrogen with deuterium,[66] indicating the absence of CM/HT.

Table 2 gives the results of the secondary KIE for transfer of a hydrogen and transfer of a deuterium in the isomerization of **1** for catalysis by acetate, WT KSI, and the mutants, along with the corresponding standard deviations. Each KIE is the average of 9−18 individual measurements. The appropriate statistical tool to determine whether there are significant differences between the isotope effects for the transfer of H and of D for a given catalyst is the t test. The results of this analysis are given in the last column of Table 2. For WT, the likelihood that the isotope effects for hydrogen transfer and deuterium transfer are different is >98% (3.3% methanol) and >99.5% (20% methanol). For D99A, it is also >99.5%, whereas for Y14F, it is >90%. For acetate catalysis, the t test was not carried out, because the KIEs are 1.031 and 1.030, respectively.

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Thus, WT and both mutants exhibit decreases in the α-secondary deuterium KIE from 1.06−1.08 for hydrogen transfer to 1.03−1.04 for deuterium transfer, which is consistent with CM/HT.

In contrast to the enzymatic reactions, the acetate-catalyzed isomerization shows no change in KIE with deuterium transfer, which is consistent with no CM/HT as part of the solution reaction. These results indicate that CM/HT is occurring in the KSI reaction, in contrast to the model acetate reaction, for which there is no evidence for CM/HT.

Previous Report of Tunneling with KSI. Xue et al.[36] previously interpreted a decrease in C-4α secondary isotope effects from 1.11 ± 0.02 (for substrate deuterated at C-4α) to 1.06 ± 0.01 (for substrate deuterated at both the C-4α and β positions) for the KSI-catalyzed isomerization of **1** in terms of a tunneling contribution to the enolization step. However, this interpretation relies on the assumption that KSI-catalyzed proton abstraction is 100% stereospecific for C-4β. Unfortunately, this assumption is not entirely valid; in fact, KSI abstracts the C-4α proton ∼5% of the time when both C-4 positions are protonated.[41] Therefore, deuteration at C-4α decreases the rate of the reaction by about 4% because of a primary KIE (assuming a primary effect of 5), similar to the amount of KIE inflation observed. Furthermore, later work[28] demonstrated that enolization is not cleanly rate-limiting, as assumed by Xue et al.,[36] which introduces further complications in the interpretation of these data. For these reasons, these results cannot be used as evidence for HT during enolization by KSI.

Normal KIE for KSI in the Absence of Tunneling. Although a contribution from tunneling can explain part of the inflated KIEs for the enzymatic proton transfer, all of the α-secondary KIEs are still normal (∼1.03−1.04) when a deuterium is being transferred (Table 2), which is still inconsistent with predictions from considerations of hybridization changes by using an unconjugated alkene as a model for the intermediate **2** (∼0.89). These results are reminiscent of the observation by Anderson[67] of an inflated α-secondary KIE for crotonase that is not decreased when deuterium is transferred instead of hydrogen.[66,67] Anderson suggested that EIEs for enolate formation may be larger (more normal) than for simple alkenes because of looser vibrational modes of the C−H bond in enolates than in simple alkenes. If this is the case, a normal KIE might be expected for protonation of an enolate ion, such as **2**. Alternatively, a contribution to the KIE from CM without tunneling could also lead to an increase in the KIE. As noted above, CM without tunneling has been calculated to lead to secondary KIEs inflations of up to 3−4%.[9,61,63]

Mechanistic Implications of Enhanced CM/HT. The finding of CM for KSI stands in contrast to the well-documented asynchronicity in nonenzymatic enolization/ketonization reactions.[68–71] In simple base-catalyzed enolate formation, proton transfer lags well behind rehybridization, leading to a TS in which the carbon is almost completely sp^3 hybridized and in which there is little resonance stabilization of the partial negative charge on the enolate carbon by the carbonyl group. In the reverse direction (ketonization), most of the resonance stabilization must be lost before the TS is reached. Thus, the TS is of higher energy because of the minimal resonance stabilization, and these reactions are slower than one might expect given the equilibrium acidity of the carbonyl compounds. The asynchronicity of the two processes, gain (or loss) of resonance stabilization and proton transfer, indicates minimal, if any, CM of the secondary proton and the transferring proton in the case of acetate catalysis. On the other hand, the observed coupling in the KSI reaction suggests that loss of resonance stabilization of the enolate ion and proton transfer are more concerted than in the acetate reaction (Figure 2), leading to a TS with greater resonance stabilization than that of the TS for acetate catalysis.

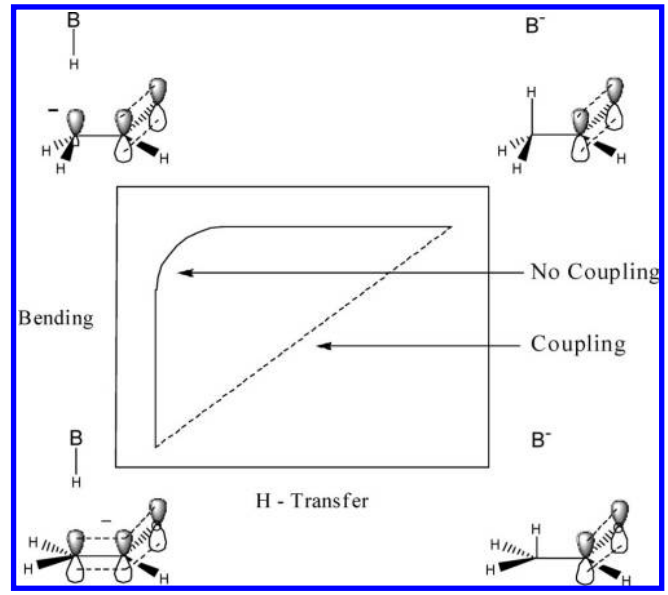

Figure 2. Diagram illustrating the increase in coupling for the KSI reaction relative to the acetate reaction. For simplicity, acetaldehyde is used as a model carbonyl compound.

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Supporting Information Available: Derivation of equations for KIE analysis by MS. This material is available free of charge via the Internet at http://pubs.acs.org.

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