Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic Chemistry 32 (2004) 341–353
BIOORGANIC CHEMISTRY
www.elsevier.com/locate/bioorg

Mini-review

Enzymatic mechanisms for catalysis of enolization: ketosteroid isomerase

Ralph M. Pollack*

Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, Baltimore, MD 21250, USA

Received 3 June 2004
Available online 29 July 2004

Abstract

Breaking a carbon–hydrogen bond adjacent to a carbonyl is a slow step in a large number of chemical reactions. However, many enzymes are capable of catalyzing this reaction with great efficiency. One of the most proficient of these enzymes is 3-oxo-Δ^5-steroid isomerase (KSI), which catalyzes the isomerization of a wide variety of 3-oxo-Δ^5-steroids to their Δ^4-conjugated isomers. In this review, the mechanism of KSI is discussed, with particular emphasis on energetic considerations. Both experimental and theoretical approaches are considered to explain the mechanistic details of the reaction.

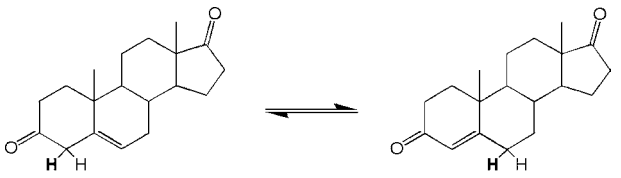
Keywords: Ketosteroid isomerase; Mechanism; Enolization; Marcus; Energetics; Structure; Transition states

1. General mechanistic considerations

Breaking a carbon–hydrogen bond adjacent to a carbonyl is a crucial step in many chemical reactions. This reaction, unlike the cleavage of bonds between hydrogen and heteroatoms, typically has a large activation barrier [1–5]. Difficulties in breaking these C–H bonds are due primarily to the instability of the anion and to the rehybridization necessary during the reaction [1–5]. However, a large number of enzymes, such as mandelate racemase [6,7], triosphosphate isomerase [8,9], citrate synthase [10,11], and 4-oxalocrotonate tautomerase [12,13], are capable of catalyzing

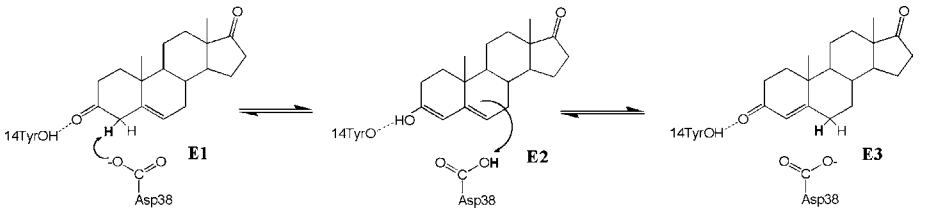
* Fax: 1-410-455-2608/1-410-455-1874.
E-mail address: pollack@umbc.edu.

0045-2068/$ - see front matter
doi:10.1016/j.bioorg.2004.06.005



1 3

Scheme 1.



Scheme 2.

this reaction with great efficiency. One of the most proficient, of these enzymes is 3-oxo-Δ^5-steroid isomerase, also known as ketosteroid isomerase (KSI) [14]. KSI catalyzes the isomerization of a wide variety of 3-oxo-Δ^5-steroids to their Δ^4-conjugated isomers (Scheme 1). In mammals, KSI is membrane bound [15], while in bacteria the enzyme is cytosolic [14]. The vast majority of mechanistic studies has been done with the soluble enzymes from *Commamonas testosteroni* (TI), previously referred to as *Pseudomonas testosteroni*, and from *Pseudomonas putida* (PI). The enzymes from these two sources are homologous (34% [16]), and structural studies have shown that the placement of the catalytic groups in the active sites is virtually identical [17]. Although there are some kinetic differences between them, it is useful to consider the evidence from both enzymes together. In this review, the numbering of the residues in the sequence from the TI enzyme will be used.

KSI is one of the most efficient enzymes known, with k_{cat}/K_m approaching the diffusion controlled limit [18] and a catalytic proficiency of greater than 10^{15} [19] for the isomerization of 5-androstene-3,17-dione (**1**) (Scheme 1). The Y14F and D38N mutants have substantially lower activity (k_{cat}) by $10^{4.7}$-fold and $10^{5.6}$-fold, respectively, implicating these groups in the catalytic mechanism [20]. A mechanism involving Asp-38 acting to transfer a proton, with proton donation by Tyr-14, was proposed (Scheme 2) [20]. To account for the large catalytic activity of KSI, it was later proposed that a short, strong (low barrier) hydrogen bond is formed between Tyr-14 and the O-3 of the steroid to facilitate proton transfer [21].

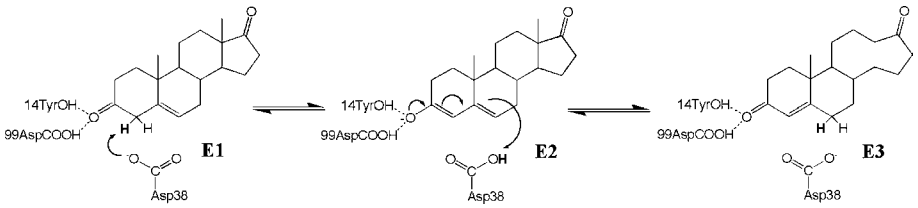
2. Structural studies

A crystal form of KSI capable of refracting to 2.7Å was reported in 1984 [22], and a 6Å structure was described in 1984 [23], but no complete atomic structure of KSI

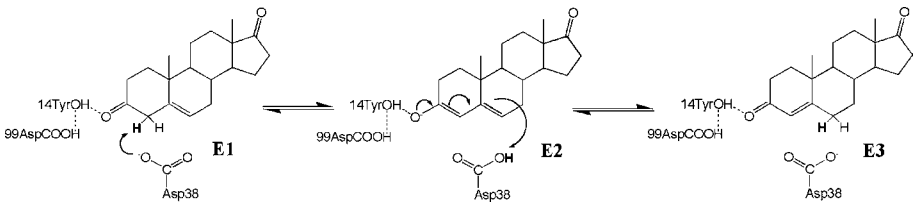
appeared until 1997, when an NMR structure was reported [24]. This structure showed that the active site is a deep hydrophobic pit with Asp-38 and Tyr-14 located at the bottom of this pit. Computational docking of a substrate molecule (**1**) into the active site, with the carbonyl oxygen within hydrogen bonding distance of Tyr-14-OH, gave a structure in which one of the carboxyl oxygens of Asp-38 is located 2.8Å above the 4β proton, in excellent position to abstract this proton. Subsequent rotation about the Cβ-COOH bond places this carboxyl proton directly above the C-6 carbon, enabling proton transfer to the β face of C-6 of the dienolate intermediate. The structure is thus entirely consistent with the previously proposed mechanistic roles of Asp-38 and Tyr-14.

The active site pit is lined with hydrophobic residues, but there is an additional ionic residue, Asp-99, located adjacent to Tyr-14 and within hydrogen bonding distance of O-3. Mutagenesis of this residue to alanine (D99A) or asparagine (D99N) results in a loss in activity (k_{cat}) at pH 7 of 3000-fold and 27-fold, respectively [24,25], implicating Asp-99 as important for enzymatic activity. A determination of the pK_a of Asp-99 gave a value of greater than 9 [25], high enough that it is still protonated at the maximum activity of KSI (pH 7–9) [26,27]. On the basis of these results, Wu et al. [24] proposed a modification of Scheme 2 that involves both Tyr-14 and Asp-99 forming hydrogen bonds directly to O-3 of the steroid (Scheme 3). This mechanism was challenged by Zhao et al. [28], who postulated a hydrogen bonding network with Asp-99 hydrogen bonding to Tyr-14, which in turn forms a hydrogen bond to O-3 (Scheme 4).

Although it is difficult to distinguish between the mechanisms of Schemes 3 and 4, there are several lines of evidence that strongly support Scheme 3 [25,29]. The first is the additivity of mutations at Tyr-14 and Asp-99 on the kinetic parameters of KSI. Kinetic experiments have shown that the rate-retarding effects of the D99A and



Scheme 3.



Scheme 4.

Y14F mutations are additive in the D99A/Y14F mutant on the rates of both the first and second steps in the chemical mechanism of the enzyme from *C. testosteroni* [29]. Similarly, the effects of analogous double mutations of residues in the *P. putida* enzyme are consistent with direct hydrogen bonding of Asp-99 and Tyr-14 to O-3 of the steroid [30]. The additivity of these effects is strong evidence for independent functioning of these two residues, as in Scheme 3 [31]. Second, a 2.3Å X-ray structure of the KSI–equilenin complex [32] confirms the hydrogen bonding network of Scheme 3. Finally, molecular dynamics simulations of KSI for both the PI [33] and the TI enzymes [34], as well as empirical valence bond simulations [35], have led to the conclusion that Scheme 3 represents the most likely arrangement of the hydrogen bonding groups at the active site.

3. Energetics of KSI catalysis

In order to evaluate the energetics, we compared the KSI reaction with a model isomerization reaction catalyzed by acetate ion, which proceeds through the same general mechanism. Thus, in both cases, a C-4 proton is abstracted to give the intermediate dienolate ion ($\mathbf{2}^-$), which is then protonated on C-6 to give product. Acetate ion is used as a model due to the carboxylate functional group, which has a similar pK_a to the active site Asp-38 in the free enzyme (pK_a 4.57 [27]) that transfers the proton. Because an understanding of an enzymatic mechanism involves more than simply "arrow pushing," detailed free energy profiles were constructed for both the nonenzymatic [36] and enzymatic reactions [37,38]. Comparison of these two free energy profiles (Fig. 1) gives insight into the catalytic strategy of KSI. The first apparent difference in the two free energy profiles is the relative stabilities of the bound

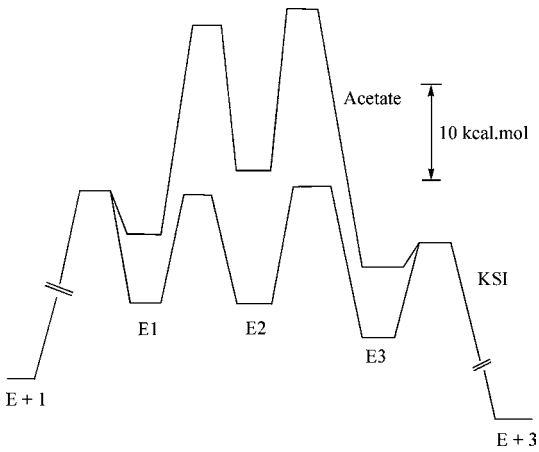


Fig. 1. The free energy profiles for the catalysis of the isomerization of **1** to **3** catalyzed by acetate and by KSI. The acetate profile assumes formation of an encounter complex with a dissociation constant of 10^{-1} M. Relative energies of bound and unbound species depend on concentration, and are not specified.

substrate and the bound intermediate in the enzymatic reaction, with **E2** and **E1** almost isoenergetic ($K_{int} \sim 1$). In contrast, the equilibrium constant in solution (K_{ext}) is approximately 10^{-8}. Thus, a major function of the enzyme is to stabilize the intermediate by ca. 11 kcal/mol.

Catalysis by KSI is also enhanced by an increase in the partitioning of the intermediate to product, relative to the acetate-catalyzed reaction. With acetate, the partitioning of the intermediate in solution is about 50:1 favoring reactant over product, whereas at the enzyme active site, partitioning is approximately 3:1. Thus, a larger fraction of the intermediate is converted to product in the enzymatic reaction than in the acetate-catalyzed reaction. Although the free energy profile shows that the second chemical step (ketonization) contributes significantly to the rate-limiting step of the reaction, Xue et al. [39] reported that there is no appreciable secondary kinetic isotope ($k_H/k_D = 1.00 \pm 0.01$) for the replacement of the C-6 hydrogen by deuterium. On this basis, they questioned the experimental results on which the free energy profile was constructed. However, in unpublished experiments, we [40] have reexamined this isotope effect, and we find that there is, in fact, a significant secondary isotope effect for replacement of H by D at this position ($k_H/k_D = 1.03 \pm 0.01$ in 3% methanol and 1.05 ± 0.02 in 20% methanol), consistent with a significant contribution of ketonization to the rate-limiting step.

The free energy profile of KSI was analyzed in the context of the general theory of Albery and Knowles [41,42], which relates the energetics of individual steps of enzymatic reactions to the catalytic ability of the enzyme. As predicted by this theory for a highly evolved reversible enzyme [41,42], the bound intermediate has the same free energy as the bound substrate. The free energy profile exhibits four kinetically significant barriers for conversion of substrate to product (substrate binding, enolization, ketonization, and product dissociation). Thus, no single step is completely rate-determining for KSI. The catalytic ability of KSI was also analyzed in terms of Marcus theory [38]. Marcus theory [43] divides the free energy of activation ($\Delta G^{\ddagger}$) into two parts: (1) the free energy of the reaction (ΔG^0) and (2) the intrinsic free energy of activation ($\Delta G^{\ddagger}_{int}$), which is the free energy of activation for a reaction in which the reactants and products are of equal stability. For the first chemical step (enolization), $\Delta G^0 = 0.7$ and $\Delta G^{\ddagger}_{int}$ is ca. 10 kcal/mol. A comparison of these values to those estimated for the acetate reaction ($\Delta G^0 = 11$ and $\Delta G^{\ddagger}_{int} =$ ca. 13 kcal/mol) reveals that the majority of the catalytic ability of KSI resides in its ability to stabilize the intermediate ($\Delta\Delta G^0 = 10$ kcal/mol, $\Delta\Delta G^{\ddagger}_{int} = 3$ kcal/mol). Thus, approximately 75% of the catalytic ability of the TI enzyme can be accounted for by stabilization of the intermediate, with the other 25% due to a decrease in the intrinsic barrier.

4. Nature of the intermediate

The first step of the mechanism for isomerization of **1** involves deprotonation of the C-4β hydrogen of the substrate by Asp-38. Although Scheme 3 depicts the function of Tyr-14 and Asp-99 as hydrogen bonding, it has been suggested [39] that proton transfer to O-3 of the steroid may be part of the reaction coordinate. Thus, this

reaction may lead to either the dienolate ion ($\mathbf{2}^-$) or the dienol (**2**). Early work [44] on spectral shifts of the intermediate analogs 17β-estradiol and 17β-dihydroequilenin when bound to KSI was interpreted in terms of the bound anion. Based upon the response of the fluorescence emission spectra to variation in pH, Zeng et al. [45] concluded that the pK_a of equilenin is perturbed from its normal pK_a of 9 in aqueous solution to ~3.5 at the active site of KSI. Subsequently, Xue et al. [46] found that mixing **1** with the D38N mutant produces a species with spectral characteristics corresponding to the dienolate ion, with some contribution by the dienol. Similar results were obtained when the dienol itself was added to D38N [38]. In these experiments, D38N is used as a model for the COOH of Asp-38, which is present in the intermediate complex.

Petrounia et al. [47,48] investigated the ionization state of the intermediate at the active site through the use of phenol and naphthol analogs. The binding constants of substituted phenols with D38N are correlated with a modified Brønsted equation that includes a hydrophobicity parameter. The high value of the Brønsted β (0.85) indicates that the negative charge in the phenol–D38N complex, and by implication on $\mathbf{2}^-$ in the KSI intermediate, is localized on the phenol ligand [47]. Similar studies on the binding of substituted naphthols to D38E gave a Brønsted β of 0.87, again leading to the conclusion that the dienolate is the preferred species [48]. In contrast, binding of these same substituted naphthols to the D38E/Y14F or D38E/D99A mutants showed a Brønsted β of only 0.28 and 0.25, respectively, indicating that the charge in these complexes does not reside on the naphthol, but rather on either Asp-99 or Tyr-14.

As discussed above, the bound intermediate has the same free energy as the bound substrate at the enzyme active site, which greatly facilitates catalysis by KSI. The reaction of **E1** → **E2** is an acid–base reaction, involving the transfer of a proton from **1**, with a pK_a of 12.7 [36], to Asp-38 (pK_a 4.57 [27]). The most obvious source of the stabilization of **E2** is the hydrogen bonding of Tyr-14 and Asp-38 to O-3 of $\mathbf{2}^-$. Since the equilibrium constant at the active site is ca. 11 kcal/mol more favorable than in aqueous solution, stabilization by hydrogen bonding would require strong hydrogen bonds if that were the only contribution to the stability of **E2**. Consistent with this theory, low barrier (short, strong) hydrogen bonds (LBHB) have been postulated to be involved in this stabilization [21,49,50], although the importance of LBHBs in KSI [29] and in enzyme mechanisms in general has been questioned [51,52].

In an effort to ascertain the nature of the stabilization of the bound intermediate relative to bound substrate, Houck and Pollack [53] determined the enthalpy and entropy changes, ΔH and $T\Delta S$, for this equilibrium for the D38E mutant of KSI.[1] The temperature dependence of K_{int} shows that both ΔH and $T\Delta S$ contribute to this equilibrium, with enthalpic considerations favoring **E2** by −6.3 kcal/mol, and entropy favoring **E1** by the same amount. The more favorable enthalpy of **E2** than **E1** can be rationalized by stabilization of the negative dienolate through the formation of hy-

[1] D38E has a similar free energy profile to wild type KSI, although in the case of D38E diffusion barriers are unimportant, making D38E an excellent choice for the determination of activation parameters.

drogen bonds between O-3 of $\mathbf{2}^-$ and both Asp-99 and Tyr-14. These hydrogen bonds are expected to be stronger to the oxyanion of $\mathbf{2}^-$ than to the neutral carbonyl oxygen of **1**. Differences in entropy between **E1** and **E2** provide support for the molecular dynamics simulations of Mazumder et al. [34] with wild type KSI, which suggest a more restricted (or "closed") complex for **E2** relative to **E1**. They suggested that this restricted motion in the **E2** complex may function to shield the active site from solvent water molecules, and thus help stabilize the protonated form of Asp-38. According to their calculations, the active site is accessible to solvent water in the **E1** complex, but not in **E2**. A restricted active site of **E2** is consistent with the greater entropy of **E1** [54,55].

In contrast to the equilibrium constant of unity at the active site of D38E, in solution the free energy of the intermediate ($\mathbf{2}^-$ + AcOH) is significantly greater than that of reactants ($\mathbf{1}$ + AcO^-) ($\Delta G^\circ = 11$ kcal/mol) [36]. The decreased stability of the intermediate is due to both an unfavorable enthalpy ($\Delta H^\circ = +7$ kcal/mol) and an unfavorable entropy ($T\Delta S^\circ = -4$ kcal/mol) [56]. Thus, the more favorable equilibrium at the active site is due almost entirely to more favorable enthalpic effects in the enzymatic case ($\Delta\Delta H^\circ =$ ca. -13 kcal/mol), with entropy differences playing only a minor role ($T\Delta\Delta S^\circ = -2.3$ kcal/mol). These results are consistent with the major driving force for stabilization of the bound intermediate being hydrogen bonds from Asp-99 and Tyr-14 to O-3 of $\mathbf{2}^-$ in the hydrophobic active site of KSI [29,32,57].

5. Structure and stabilization of the chemical transition state(s)

Although the majority of the catalytic power for the first step of the KSI reaction comes from stabilization of the intermediate through a lowering of ΔG^0 by ca. 10 kcal/mol, there is also a significant effect on the intrinsic activation barrier ($\Delta\Delta G^{\ddagger}_{int} = 3$ kcal/mol) compared to the acetate-catalyzed reaction. This $\Delta G^{\ddagger}_{int}$ effect can be interpreted as a specific interaction with the transition state that does not exist in either the bound substrate or the bound intermediate.

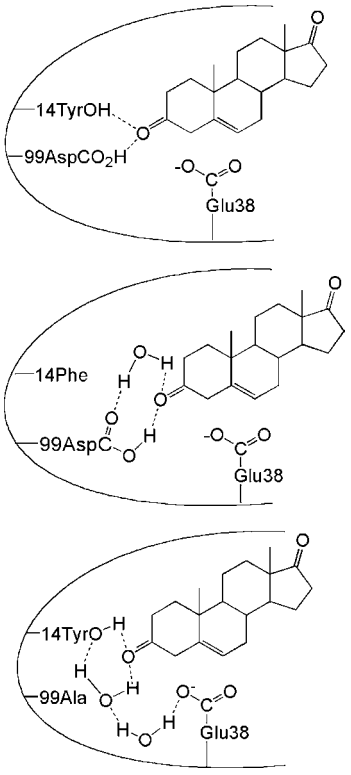
How then does KSI stabilize the transition state with an interaction specific to it? Warshel [58,59] and others [35,60] have noted the importance of a preorganized active site that is complementary to the transition state in enzyme catalysis. Using empirical valence bond simulations of the PI enzyme, Feierberg and Åqvist [35] concluded that 60% of the catalytic activity is due to stabilization by hydrogen bonding, and 40% is due to a reduction in the reorganization energy. This preorganized active site avoids the energy needed to reorient solvent dipoles, which is required in the corresponding solution reaction. Thus, the active site hydrogen bonds are already oriented towards O-3 in the enzyme–substrate complex, and the kinetic barrier associated with solvent reorganization is reduced or eliminated. This type of solvent reorganization has been postulated to be an important determinant of the sluggishness of enolization reactions in aqueous solution [2–4]. In these reactions, solvation of the anionic oxygen is thought to "lag" behind proton transfer, resulting in less than optimal solvation of the transition state.

Analysis of the temperature dependence of the rate constants for the D38E mutant of KSI shows that proton abstraction from C-4 of bound **1** (**E1**) by Glu-38 of D38E is characterized by both an unfavorable entropy term ($T\Delta S^{\ddagger} = -6.3$ kcal/mol) and an unfavorable enthalpy term ($\Delta H^{\ddagger} = +7.6$ kcal/mol) [53]. Surprisingly, the change in entropy on going from **E1** to the transition state for proton abstraction (**TS1**) is identical to that on going from **E1** to **E2**. One might expect that the partial bond formation between Glu-38 and the hydrogen being transferred at the transition state would restrict rotation of the carboxylate functional group of Glu-38, resulting in a greater entropic penalty at the transition state than at the intermediate, where that functional group is free to rotate.

It is of interest to compare the activation parameters for both the enzymatic (k_{cat}/K_m) and the acetate-catalyzed deprotonation of **1**. Proton abstraction by acetate ion from **1** has an enthalpy of activation of 16.4 kcal/mol, similar to that observed for other proton abstractions from carbon atoms α to a carbonyl in solution [56]. At the active site of D38E, $\Delta H^{\ddagger}$ is reduced by ca. 13 kcal/mol, presumably due to strengthening of the hydrogen bonds with Asp-99 and Tyr-14 as O-3 becomes progressively more charged [61]. In addition, the active site of KSI provides a hydrophobic environment, in which hydrogen bonds are expected to be stronger than in an aqueous environment [62]. Entropies of activation for the two second-order reactions, however, are similar ($T\Delta S^{\ddagger} =$ ca -5 kcal/mol). A comparison of these entropies of activation is difficult since several factors are at work, such as loss of translational entropy, restriction of the enzyme, loss of rotational entropy of the COO^- and restriction of solvent in the acetate reaction.

As a probe for whether preorganization of the active site hydrogen bonds is important for KSI catalysis, we determined activation parameters for the D38E/Y14F and D38E/D99A mutants to compare to those for D38E itself [63]. The mutants D38E/Y14F and D38E/D99A show reduced catalytic activity by ca $10^{4.4}$-fold and $10^{3.4}$-fold, respectively [48]. The most straightforward explanation for the reduced rates is that in each mutant a hydrogen bonding group at the active site is being eliminated, which should be exhibited as an increase in $\Delta H^{\ddagger}$. However, a comparison of the overall activation parameters for the D38E/Y14F and D38E/D99A mutants with those for the D38E enzyme shows that in each case the loss of activity is due to entropy changes, rather than changes in enthalpy [63]. Thus, $T\Delta S^{\ddagger}$ (k_{cat}/K_M) is approximately 7 kcal/mol less favorable for both of the double mutants, while $\Delta H^{\ddagger}$ actually decreases by 3.1 kcal/mol (D38E/D99A) and 1.3 kcal/mol (D38E/Y14F).

Although the activation parameters have yet to be determined for each of the microscopic rate constants of the double mutants D38E/Y14F and D38E/D99A, comparisons can be made of the ketonization transition states (**TS2**). Since the second chemical step (reprotonation) is entirely rate limiting for the double mutants [57], the activation parameters for k_{cat}/K_M correspond to conversion of **E** + **1** to **TS2** for both D38E/Y14F and D38E/D99A. The lack of a significant increase in the enthalpy of activation for the mutants in which a hydrogen bonding group has been eliminated from the active site suggests that these hydrogen bonds have been replaced by other hydrogen bonds. The large decrease in the entropy of activation is consistent with restricted motion of additional water molecules at the active site that



Scheme 5.

provide these new hydrogen bonds. Possible structures consistent with this hypothesis are shown in Scheme 5. In these structures, these additional water molecules are depicted in the holes left by the missing oxygens of Asp-99 or Tyr-14.

6. Partitioning of the intermediate

The ability of KSI to catalyze the reaction of the intermediate to product rather than to reactant is a significant feature of the reaction mechanism [18], and provides a factor of about 20-fold of the catalytic rate enhancement over the acetate-catalyzed reaction. One might initially suspect that this enhancement is due to positioning of the carboxylate of Asp-38 such that it has increased access to C-6 compared to C-4. However, changing the position of this group through mutation to glutamate (D38E) has little effect on the partitioning [64]. In contrast, mutations at the hydrogen bonding site that eliminate one or both of the hydrogen bonding groups (Y14F, D99A, and Y14F/D99A), that change the position (D99E), or that change the hydrogen bonding effectiveness (D99N) all modify the partitioning ratio, so that the intermediate reverts almost exclusively to substrate [57]. A possible rationale for these

results is that the O-3 of the steroid is in a different position in the transition state for protonation at C-4 than it is for protonation at C-6. If the hydrogen bonds from Tyr-14 and Asp-99 are optimally positioned for protonation at C-6 by Asp-38, then hydrogen bonding from these groups might be weaker at the transition state for protonation at C-4. Elimination or weakening of one or both of these hydrogen bonds should have more effect on **TS2** than on **TS1**. Another explanation for these results is that the position of Asp-38 may change relative to C-4 and C-6 in the mutant proteins. However, it has been found that, for the PI enzyme, the electron density in the crystal structures of the wild type, the Y14F mutant, and the D99L mutant shows no significant difference [65].

An alternate model to explain the increased stability of **TS2** relative to **TS1** comes from the difference in enthalpies and entropies of activation for protonation of **E2** at C-4 (**TS1**) and at C-6 (**TS2**) in the D38E mutant [53]. For this mutant, the $\Delta G^{\ddagger}$s for formation of **TS1** and **TS2** are the same, but **TS1** is favored over **TS2** by 2 kcal/mol in $\Delta H^{\ddagger}$, whereas **TS2** is favored by 2 kcal/mol in $T\Delta S^{\ddagger}$. In contrast, protonation of $\mathbf{2}^{-}$ by acetic acid in solution favors formation of **1**, which is due primarily to a more favorable entropy of activation ($T\Delta\Delta S^{\ddagger} = 3.5 \pm 1.0$ kcal/mol). Enthalpy differences for the two processes are negligible within a relatively large error limit ($\Delta\Delta H^{\ddagger} = 1.2 \pm 2.4$ kcal/mol). Thus, it appears that the ability of D38E to change the partitioning ratio is primarily due to a more favorable entropy of **TS2** than of **TS1**.

This result is surprising in view of our previous explanation for preferential enzymatic stabilization of **TS2** by changes in hydrogen bond strengths, which would be expected to show up as a difference in $\Delta H^{\ddagger}$ [57]. A possible explanation for the entropy effect may be found in a consideration of hydration differences of the active site at the two transition states (**TS1** and **TS2**). Mazumder et al.'s calculations indicate that during the conversion of **E1** to **E2** water is released from the active site, and that the reaction of **E2** to **E1** involves the reentry of water into the active site [34]. In addition, Feierberg and Åqvist [35] found that the conversion of **E2** to **E3** is best simulated when no water molecule is present in the active site in either **E2** or **E3** for KSI from PI. Thus, for **TS1** there is an entropic cost due to water reentering the active site, but there is no such penalty at **TS2**. The entropy for release of a water molecule from an active site is ca. +2–3 kcal/mol [54,55], and may account for the entropy differences between **TS1** and **TS2**. This explanation, however, does not account for the effects of the hydrogen bonding mutations on the partitioning ratio.

7. How does KSI maintain the pK_a of Asp-38 in the hydrophobic active site?

The catalytic base, Asp-38, is located in a deep hydrophobic pit with no polar interactions [24]. Nevertheless, in the **E1** complex (TI) this group exhibits a normal pK_a value (4.75), which is shifted only slightly from its value in the free enzyme (4.57) [27]. From a catalytic perspective, it is important that binding of substrate to KSI not shift this pK_a significantly, since if the carboxyl group of Asp-38 is protonated in the Michaelis complex, it will lose its ability to abstract a proton from **1**. Since

the active site is very hydrophobic [24], it is surprising that the pK_a is not shifted several units upward. A possible solution to this problem has been provided by molecular dynamics simulations of both the TI enzyme and the PI enzyme. For TI, these simulations predict that Asp-38 maintains either two or three hydrogen bonds with water molecules in the **E1** complex [34]. In **E2** complex, however, the active site is shielded from bulk water, which is consistent with the experimental observation that proton transfer from C-4 to C-6 is predominantly intramolecular [66–68]. Similar results were found with the PI enzyme [35], and it was calculated that the absence of water in the **E1** complex would perturb the pK_a of Asp-38 by about 5 units.

8. Conclusions

It has been almost 50 years since the first ketosteroid isomerase activity was described by Talalay and Wang [14], and in that time quite a few mechanistic proposals have been put forward. In the early years, before the advent of site-directed mutagenesis and in the absence of an atomic level structure, it was difficult to identify the functional groups that are involved in catalysis. However, the detailed chemical mechanism, including the nature of the hydrogen bonding network, now appears to be on firm ground, although the importance, of a low barrier hydrogen bond has not yet been satisfactorily resolved. Nevertheless, the ability to push electrons around in a reaction mechanism is only a start to elucidating the catalytic strategy of an enzyme. It is also important that the energetics of enzymatic reactions be determined and compared with an appropriate nonenzymatic reaction to ascertain what catalytic principles are being utilized. With KSI, this approach has enabled us to understand quite a bit of the more subtle mechanistic details. Thus, both theoretical and experimental approaches have yielded evidence that the existence of a preorganized active site is important in selective stabilization of the transition states. The enzyme-intermediate complex is stabilized by hydrogen bonding, as expected, but also by constriction of the enzyme to force water out of the active site, which increases the basicity of Asp-38 relative to the enzyme–substrate complex. We also know that the partitioning of the intermediate on the enzyme surface has been modified from that in the model reaction, but the exact manner in which this is accomplished is still unclear.

Acknowledgments

Work in the author's laboratory on KSI has been supported by grants from the NIH (GM 33059, GM 38155 and GM 49082).

References

[1] J.R. Keeffe, A.J. Kresge, in: Z. Rappoport (Ed.), The Chemistry of Enols, Wiley, Chichester, England, 1990 (Chapter 7).

[2] C.F. Bernasconi, Acc. Chem. Res. 25 (1992) 9–16.
[3] C.F. Bernasconi, P.J. Wenzel, J. Am. Chem. Soc. 118 (1996) 11446–11453.
[4] C.F. Bernasconi, Adv. Phys. Org. Chem. 27 (1992) 119–238.
[5] C.F. Bernasconi, M. Panda, M.W. Stronach, J. Am. Chem. Soc. 117 (1995) 9206–9212.
[6] M. St. Maurice, S.L. Bearne, Biochemistry 43 (2004) 2524–2541.
[7] G.L. Kenyon, J.A. Gerlt, G.A. Petsko, J.W. Kozarich, Acc. Chem. Res. 28 (1995) 178–186.
[8] T.L. Amyes, A.C. O'Donoghue, J.P. Richard, J. Am. Chem. Soc. 123 (2001) 11325–11326.
[9] J.R. Knowles, W.J. Albery, Acc. Chem. Res. 10 (1977) 105–111.
[10] W. Yang, D.G. Drueckhammer, J. Phys. Chem. B 107 (2003) 5986–5994.
[11] L.C. Kurz, G. Drysdale, M. Riley, M.A. Tomar, J. Chen, R.J.M. Russell, M.J. Danson, Biochemistry 39 (2000) 2283–2296.
[12] S.C. Wang, W.H. Johnson Jr., C.P. Whitman, J. Am. Chem. Soc. 125 (2003) 14282–14283.
[13] G.A. Cisneros, H. Lu, Y. Zhang, W. Yang, J. Am. Chem. Soc. 125 (2003) 10384–10393.
[14] P. Talalay, V.S. Wang, Biochem. Biophys. Acta 18 (1955) 300–301.
[15] S. Murota, C.C. Fenselau, P. Talalay, Steroids 17 (1971) 25–37.
[16] S.W. Kim, K.Y. Choi, J. Bacteriol. 177 (1995) 2602–2605.
[17] H.-S. Cho, G. Choi, K.Y. Choi, B.-H. Oh, Biochemistry 37 (1998) 8325–8330.
[18] D.C. Hawkinson, T.C.M. Eames, R.M. Pollack, Biochemistry 30 (1991) 10849–10858.
[19] A. Radzicka, R. Wolfenden, Science 267 (1995) 90–93.
[20] A. Kuliopulos, A.S. Mildvan, D. Shortle, P. Talalay, Biochemistry 28 (1989) 149–159.
[21] Q. Zhao, C. Abeygunawardana, P. Talalay, A.S. Mildvan, Proc. Natl. Acad. Sci. USA 93 (1996) 8220–8224.
[22] E.M. Westbrook, J. Mol. Biol. 103 (1976) 659–664.
[23] E.M. Westbrook, E.O. Piro, P.B. Sigler, J. Biol. Chem. 259 (1984) 9096–9103.
[24] Z.R. Wu, S. Ebrahimian, M.E. Zawrotny, L.D. Thornburg, G.C. Perez-Alvarado, P. Brothers, R.M. Pollack, M.F. Summers, Science 276 (1997) 415–418.
[25] L.D. Thornburg, F. Hénot, D.P. Bash, D.C. Hawkinson, S.D. Bartel, R.M. Pollack, Biochemistry 37 (1998) 10499–10506.
[26] H. Weintraub, A. Alfsen, E.-E. Baulieu, Eur. J. Biochem. 12 (1970) 217–221.
[27] R.M. Pollack, S. Bantia, P.L. Bounds, B.D. Koffman, Biochemistry 25 (1986) 1905–1911.
[28] Q. Zhao, C. Abeygunawardana, A.G. Gittis, A.S. Mildvan, Biochemistry 36 (1997) 14616–14626.
[29] R.M. Pollack, L.D. Thornburg, Z.R. Wu, M.F. Summers, Arch. Biochem. Biophys. 370 (1999) 9–15.
[30] G. Choi, N.-C. Ha, S.W. Kim, D.-H. Kim, S. Park, B.-H. Oh, K.Y. Choi, Biochemistry 39 (2000) 903–909.
[31] A.S. Mildvan, D.S. Weber, A. Kuliopulos, Arch. Biochem. Biophys. 294 (1992) 327–340.
[32] H.-S. Choi, N.-C. Ha, G. Choi, H.-J. Kim, D. Lee, K.S. Oh, K.S. Kim, W. Lee, K.Y. Choi, B.-H. Oh, J. Biol. Chem. 274 (1999) 32863–32868.
[33] H. Park, K.M. Merz Jr., J. Am. Chem. Soc. 125 (2003) 901–911.
[34] D. Mazumder, K. Kahn, T.C. Bruice, J. Am. Chem. Soc. 125 (2003) 7553–7561.
[35] J. Feierberg, J. Åqvist, Biochemistry 41 (2002) 15728–15735.
[36] B. Zeng, R.M. Pollack, J. Am. Chem. Soc. 113 (1991) 3838–3842.
[37] T.C.M. Eames, D.C. Hawkinson, R.M. Pollack, J. Am. Chem. Soc. 112 (1990) 1996–1998.
[38] D.C. Hawkinson, R.M. Pollack, N.P. Ambulos, Biochemistry 33 (1994) 12172–12183.
[39] L. Xue, P. Talalay, A.S. Mildvan, Biochemistry 29 (1990) 7491–7500.
[40] T.C. Wilde, R.M. Pollack, unpublished results.
[41] W.J. Albery, J.R. Knowles, Biochemistry 15 (1976) 5627–5631.
[42] W.J. Albery, J.R. Knowles, Biochemistry 15 (1976) 5631–5640.
[43] A.O. Cohen, R.A. Marcus, J. Phys. Chem. 72 (1968) 4249–4256.
[44] S.-F. Wang, F.S. Kawahara, P. Talalay, J. Biol. Chem. 238 (1963) 576–585.
[45] B. Zeng, P.L. Bounds, R.F. Steiner, R.M. Pollack, Biochemistry 31 (1992) 1521–1528.
[46] L. Xue, A. Kuliopulos, A.S. Mildvan, P. Talalay, Biochemistry 30 (1991) 4991–4997.
[47] I.P. Petrounia, R.M. Pollack, Biochemistry 37 (1998) 700–705.
[48] I.P. Petrounia, G. Blotny, R.M. Pollack, Biochemistry 39 (2000) 110–116.

[49] W.W. Cleland, P.A. Frey, J.A. Gerlt, J. Biol. Chem. 273 (1998) 25529–25532.
[50] A.S. Mildvan, M.A. Massiah, T.K. Harris, G.T. Marks, D.H.T. Harrison, C. Viragh, P.M. Reddy, I.M. Kovach, J. Mol. Struct. 26 (2002) 163–175.
[51] C.L. Perrin, J.B. Nielson, Annu. Rev. Phys. Chem. 48 (1997) 511–544.
[52] P.A. Kollman, B. Kuhn, O. Donini, M. Perakyla, R. Stanton, D. Bakowies, Acc. Chem. Res. 34 (2001) 72–79.
[53] W.J. Houck, R.M. Pollack, unpublished results.
[54] J.D. Dunitz, Science 264 (1994) 670.
[55] S.J. Suresh, V.M. Naik, J. Chem. Phys. 113 (2000) 9727–9732.
[56] W.J. Houck, R.M. Pollack, J. Am. Chem. Soc. 125 (2003) 10206–10212.
[57] L.D. Thornburg, Y.R. Goldfeder, T.C. Wilde, R.M. Pollack, J. Am. Chem. Soc. 123 (2001) 9912–9913.
[58] A. Yadav, R.M. Jackson, J.J. Holbrook, A. Warshel, J. Am. Chem. Soc. 113 (1991) 4800–4805.
[59] A. Warshel, J. Biol. Chem. 273 (1998) 27035–27038.
[60] W.R. Cannon, S.J. Benkovic, J. Biol. Chem. 273 (1998) 26257–26260.
[61] S.-O. Shan, D. Herschlag, J. Am. Chem. Soc. 118 (1996) 5515–5518.
[62] S.-O. Shan, D. Herschlag, Proc. Natl. Acad. Sci. USA 93 (1996) 14474–14479.
[63] W.J. Houck, R. Dodson, R.M. Pollack, unpublished results.
[64] K.S. Oh, S.-S. Cha, D.-H. Kim, H.-S. Cho, N.-C. Ha, G. Choi, J.Y. Lee, P. Tarakeshwar, H.S. Som, K.Y. Choi, B.-H. Oh, K.S. Kim, Biochemistry 39 (2000) 13891–13896.
[65] M.E. Zawrotny, R.M. Pollack, Biochemistry 33 (1994) 13896–13902.
[66] S.K. Malhotra, H.J. Ringlod, J. Am. Chem. Soc. 87 (1965) 3228–3236.
[67] A. Viger, S. Coustal, A. Marquet, J. Am. Chem. Soc. 103 (1981) 451–458.
[68] M.E. Zawrotny, D.C. Hawkinson, G. Blotny, R.M. Pollack, J. Biochem. 35 (1996) 6438–6442.