Selective Catalysis of Elementary Steps by Asp-99 and Tyr-14 of 3-Oxo-Δ^5-Steroid Isomerase

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3-Oxo-Δ^5-steroid isomerase (KSI, EC 5.3.3.1) from *Pseudomonas testosteroni* catalyzes the isomerization of a variety of Δ^5-unsaturated 3-oxosteroids (e.g., **1**) to their more stable conjugated Δ^4-isomers (e.g., **3**). This enzyme is one of the most active and efficient ones known, with a catalytic efficiency of greater than 10^{15}.[1] It is generally accepted that catalysis is effected by Asp-38 acting to transfer a proton from C-4 to C-6, while both Tyr-14 and Asp-99 stabilize the intermediate and the flanking transition states.[2] On the basis of the solution structure of KSI, we proposed that these two electrophiles function through direct hydrogen bonding to O-3 of the steroid (Scheme 1).[3] This mechanism is supported by both mutagenesis and structural evidence.[4]

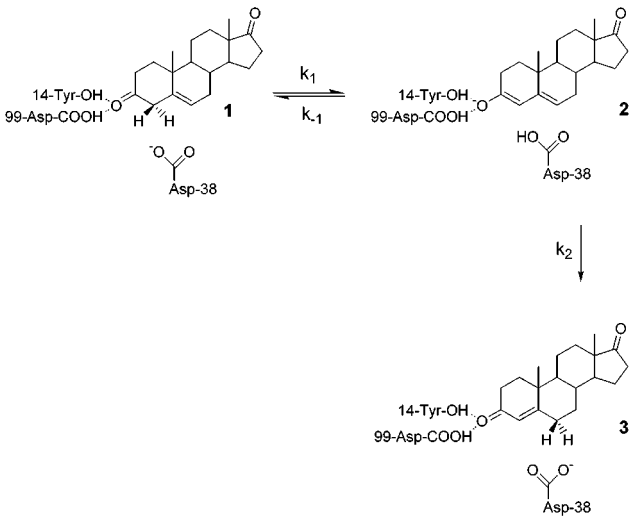
A comparison of the free energy profiles of the isomerization of 5-androstene-3,17-dione (**1**) to 4-androstene-3,17-dione (**3**) by KSI and by acetate ion shows that KSI stabilizes the transition state for the second step of the isomerization (TS2) more efficiently than it does the transition state for the first step (TS1).[5] Thus, in the nonenzymatic reaction, TS2 is approximately 1.7 kcal/mol higher in energy than TS1, but in the KSI-catalyzed reaction the difference is only about 0.5 kcal/mol. This result is in accord with the theory of Albery and Knowles that predicts similar energies for these transition states for an evolutionarily optimal enzyme.[6]

However, the source of the ability of KSI to discriminate between these two very similar structures has been unclear. From results reported here, we conclude that this differential stabilization is due to preferential recognition of the second transition state by the hydrogen-bonding groups Tyr-14 and Asp-99 at the active site.

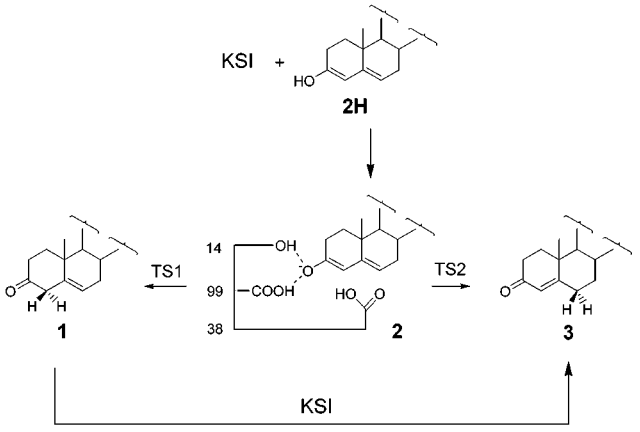
To determine the effect of each of these electrophilic groups on the relative stabilities of the two transition states, we examined the reaction of the dienol **2H** with mutants of KSI in which the hydrogen-bonding groups were removed (the D99A and Y14F mutants, as well as the double mutant Y14F/D99A). We have previously shown[5b,7] that **2H** can be generated transiently in a sequential mixing stopped-flow apparatus and that the reaction of this species with KSI can be monitored by UV spectroscopy (Scheme 2). With wild-type KSI (WT), there is a rapid first-order decay of **2H** to a mixture of **1** and **3**, followed by a slower conversion of **1** to **3**. We have interpreted this reaction in terms of a fast proton transfer from the OH of the dienol to the COO^- of Asp-38, to generate an intermediate that is identical to the intermediate along the normal reaction pathway for conversion of **1** to **3** (Scheme 2). This intermediate then partitions to a mixture of reactant (**1**) and product (**3**), resulting in a drop in absorbance. The subsequent rise in absorbance is due to KSI-catalyzed conversion of the initially formed **1** to **3.** Analysis of the extent of loss of absorbance in the initial drop gives the partitioning ratio of this reaction, which leads to the relative energies of the transition states for protonation at C-4 (TS1) and at C-6 (TS2).

With WT, the initial ratio of products ([**1**]/[**3**]) at pH 7 varies from 0.6 to 3.0, depending on solvent composition (2.5−20% methanol),[5b] consistent with similar barriers to protonation at C-4 (TS1) and at C-6 (TS2). If the catalyzing base (Asp-38) is replaced by glutamate (D38E mutant), there is little change in the partitioning ratio (1.0), although the overall rate constant (k_{cat}) for isomerization of **1** to **3** is decreased by ca. 300-fold.[8]

Scheme 1



Scheme 2



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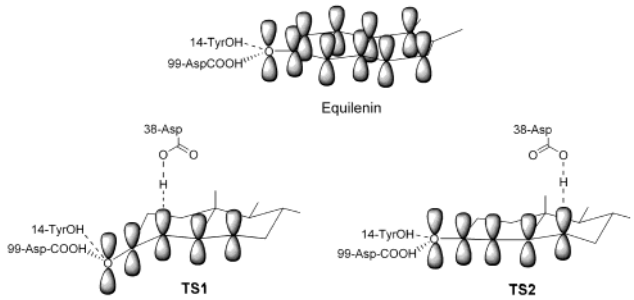
The results differ dramatically when similar experiments are run with mutants that lack one or both of the hydrogen-bonding groups Tyr-14 or Asp-99. With the D99A mutant,[9] which lacks the carboxyl group of Asp-99 and is ca. 3000-fold less active than WT (k_{cat}),[3] a pseudo-first-order decay is again observed, but the reaction of **2H** produces exclusively **1**, with no detectable **3** formed (<5%). Similarly, reaction of **2H** with the Y14F mutant, which is 50 000-fold less active than WT (k_{cat}),[2c] also produces exclusively **1** within the limits of detection. The double mutant Y14F/D99A, which we found to be 10^8-fold less active than WT, also gives solely **1** as the product of partitioning. Therefore, deletion of either Tyr-14 or Asp-99 (or both) has a greater destabilizing effect on the transition state for protonation of **2** at C-6 (TS2) than that for protonation at C-4 (TS1). For each of these mutants, the partitioning ratio is >20-fold favoring **1** over **3**.

In previous work, we determined the free energy profiles of the nonenzymatic isomerization of **1** by hydroxide and by acetate ion. In the presence of either hydroxide ion or acetate ion, isomerization occurs through a dienolate intermediate (**2**), similar to the mechanism utilized by KSI. In the absence of enzyme, **2** is preferentially converted to **1** with little **3** formed (96 and 98% **1**, respectively).[5a,10] Thus, during each of these model reactions, the large majority of intermediate is reprotonated on C-4 to regenerate **1**, and only a small fraction (2−4%) is protonated at C-6 to form **3**, in contrast to the KSI reaction. However, the partitioning with the mutants is similar to that with the model reactions.

To confirm the importance to partitioning of the identity of the electrophilic groups that interact with the incipient anion at O-3, the mutants D99N and D99E were also examined. In the former, hydrogen bonding is weakened by a decrease in the acidity of the electrophile (k_{cat} decrease ca. 30-fold);[4a] in the latter, hydrogen bonding is weakened by a change in the position of the COOH (k_{cat} decrease ca. 60-fold). With both mutants, partitioning is again completely toward reactant (**1**) within experimental error. Thus, the partitioning of the intermediate dienolate ion (**2**) is controlled by the nature of the hydrogen-bonding groups that interact with O-3 of the steroid.

A possible explanation for the effect of these mutations on the C-4/C-6 protonation ratio is that the COOH group of Asp-38 is positioned somewhat closer to C-4 than to C-6 in the mutant proteins than in WT. This difference could lead to the increased ratio of protonation at C-4 relative to that of C-6 in the mutants. Evidence against this interpretation comes from the similarity of the partitioning ratio for WT and D38E, in which the COOH is moved significantly from that of WT. Also, the rate constant (k_{cat}) for the D99N mutant is ca. 100-fold larger than for D99A, and 30-fold smaller than WT. Since protonation at C-6 is rate-limiting in both mutants, there must be a significant effect on the energy of this transition state from the D99N hydrogen bond. Consequently, it appears that the hydrogen bond from residue 99 to the steroid oxygen remains intact, suggesting that there is no major reorientation of the steroid at the active site. Finally, the additivity of the effect on log k_{cat} of the Y14F and D99A mutations in the Y14F/D99A mutant is good evidence that there is no change in the position of the hydrogen bonds in the single mutants.

Scheme 3



Rationalization of the ability of the hydrogen-bonding groups to discriminate between these two very similar transition states can be found in the work of Whalen and co-workers on the base-catalyzed isomerization of 3-cyclopentenone and of 3-cyclohexenone.[11] The intermediate dienolate ion from 3-cyclohexenone is protonated at the α-carbon 575-fold faster than at the γ-carbon, whereas the corresponding ratio for the ion from 3-cyclopentenone is 3-fold. These authors attributed this difference to reduced overlap of the double bonds in the dienolate of 3-cyclohexenone, destabilizing the transition state for protonation at the γ-carbon due to decreased orbital overlap in the transition state. From this result, it can be inferred that protonation at C-6 of the dienolate intermediate (**2**) relative to protonation at C-4 would be accelerated by stabilization of a transition state in which all of the p-orbitals are coplanar.

The 2.26 Å crystal structure of KSI complexed with the intermediate analogue equilenin[4b] shows that the hydrogen bonds from both Tyr-14-OH and Asp-99-COOH to O-3 lie directly in the plane of the steroid A and B rings with distances of 2.58 ± 0.08 Å and 2.62 ± 0.07 Å, respectively (Scheme 3). Because TS2 leads to a conjugated system, conjugation is maintained, and TS2 should have a nearly planar structure, similar to that of equilenin. Consequently, these hydrogen bonds should be very effective in stabilizating TS2. In contrast, in TS1, puckering of the A ring by protonation at C-4, will lengthen the hydrogen bonds, resulting in weaker interactions. Therefore, stabilization of TS2 by hydrogen bonding should be more effective than stabilization of TS1.

The ability to discriminate between two transition states on the basis of a subtle difference in the positions of the oxygen atoms has enabled KSI to attain its present state of efficiency. As part of its catalytic strategy, KSI has evolved such that the higher-energy transition state is stabilized more than the lower-energy one, giving a partitioning ratio approaching unity and leading to a nearly optimal enzyme.

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(9) All mutants were either available from previous work[3,4a] or were prepared as previously described.[4a] To verify the desired mutation was present, the entire KSI gene of each mutant was sequenced by the Biopolymer Laboratory at the University of Maryland, Baltimore. No other mutations were present except for the D99A single mutant[4a] and the Y14F/D99A double mutant, which also had a silent mutation at Pro-97. Proteins (except D99E) were expressed in DH5α *Escherichia coli* grown in 2YT medium, and purified as described previously,[4a] except that 55% saturation with ammonium sulfate was required to precipitate the Y14F/D99A protein. The D99E mutant was expressed in Epicurean Coli XL1-Blue with 2YT medium. The resulting proteins were homogeneous by SDS-PAGE. Steady-state kinetic parameters were determined in 34 mM phosphate (pH 7.0) or 260 mM TES (pH 7.3) in solutions with methanol concentrations between 3 and 30%. Partitioning ratios were obtained in 260 mM TES buffer at pH 7.3 or 330 mM phosphate at pH 7.0 (D99E) and 25°. Relative rate constants of the various mutants differ somewhat as a function of methanol concentration in the solution, but partitioning ratios show no apparent change.

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