Supporting Information

Ground State Destabilization From a Positioned General Base in the Ketosteroid Isomerase Active Site

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Supporting Text

Prior structural studies of pKSI and the structural studies of tKSI reported herein indicate that bound steroid ligands can occupy two conformations (*59, 60*). Ligands can bind in a forward conformation with the A-ring situated near the general base and the D-ring solvent exposed (a productive conformation), or in a nonproductive backward conformation with the D-ring situated near the general base and the A-ring solvent exposed. To test if the ligands used herein bind with the A-ring near the general base, as needed for the KSI isomerization reaction, we compared the binding affinities of ligands with identical A-rings, but with a hydroxyl, sulfate, or hemisuccinate substituent on the D-ring (Figure S3A). If the ligands bind with the A-ring near residue 38, the binding affinities for the 19-NT analogs would be expected to be the same, but if the ligands bind with the D-ring situated near residue 38, the binding affinities for the 19-NT analogs would be expected to differ. The results in Figure S3B show that ligands with different D-ring substituents have similar binding affinities for each Asp38 mutant. Further, each ligand shows a similar increase in binding affinity upon introduction of the Asp38Asn and Asp38Ala mutations (Tables S2 and S3). These results strongly suggest that the ligands bind with the A-ring near residue 38 in solution under our assay conditions.

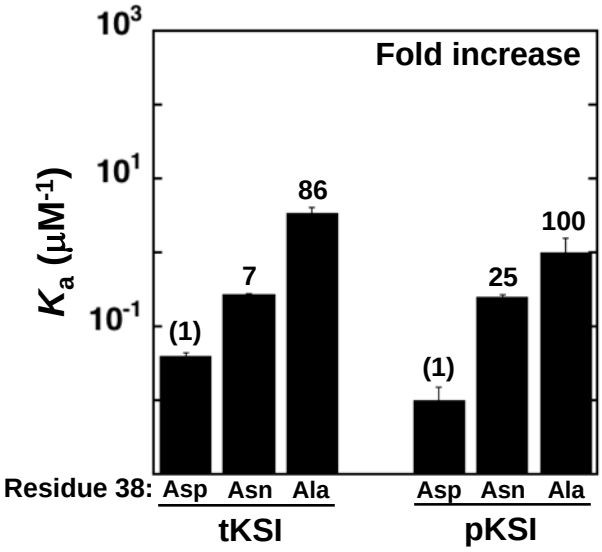

Figure S1. Effects of Asp38 mutations on 19-nortestosterone association constants (K_a). Values are from Table S1.

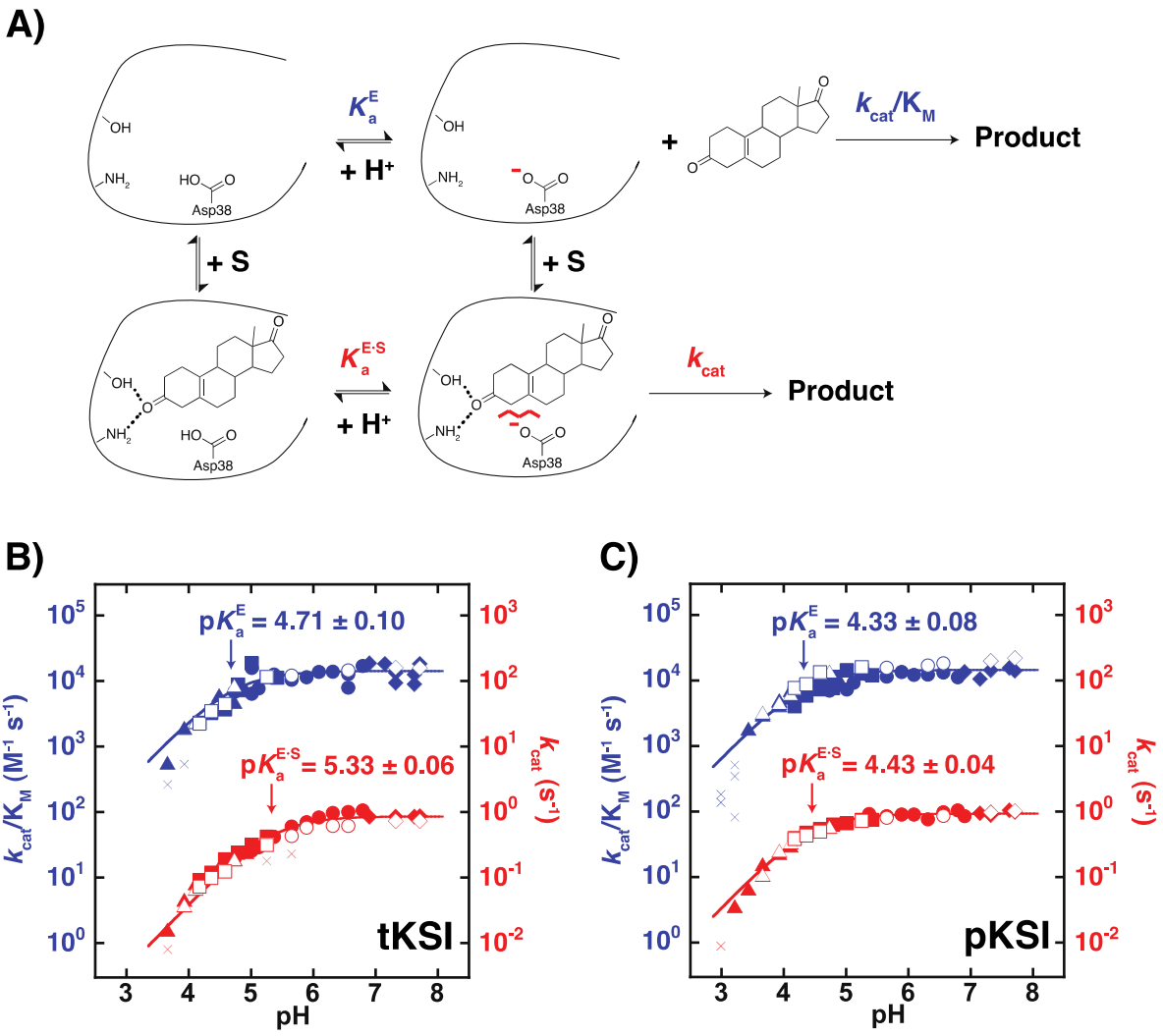

Figure S2. pH dependencies of k_{cat}/K_M and k_{cat} for tKSI and pKSI Asp99Asn with 5(10)estrene-3,17-dione. A) Reaction scheme showing that if the abutment of anionic Asp38 against bound substrate is destabilizing, the pK_a of the enzyme-substrate complex (E·S) is predicted to be higher than the pK_a of free enzyme (E). B, C) Determinations of k_{cat}/K_M (blue) and k_{cat} (red) for tKSI (B) and pKSI (C) Asp99Asn. Data were collected and analyzed as described in Experimental Procedures. The pK_a value from each fit (to a single titration) is depicted next to each titration curve with the standard error from regression analysis. Open and closed symbols represent substrate concentrations of 2.5 μM and 5 μM used to determine k_{cat}/K_M, and 300 μM and 600 μM used to determine k_{cat} respectively. Prior work establishes K_M values of 20-50 μM

for these enzymes (*44)*. Buffers used were sodium formate (triangles), sodium acetate (squares), Na•MES (circles) and Na•MOPS (diamonds). Points omitted from the fits are depicted as ×'s. Without omitting these points, the resulting pK_a values are 4.72 ± 0.10 (k_{cat}/K_M) and 5.41 ± 0.07 (k_{cat}) for tKSI, and 4.34 ± 0.07 (k_{cat}/K_M) and 4.42 ± 0.04 (k_{cat}) for pKSI, values that do not change the conclusions.

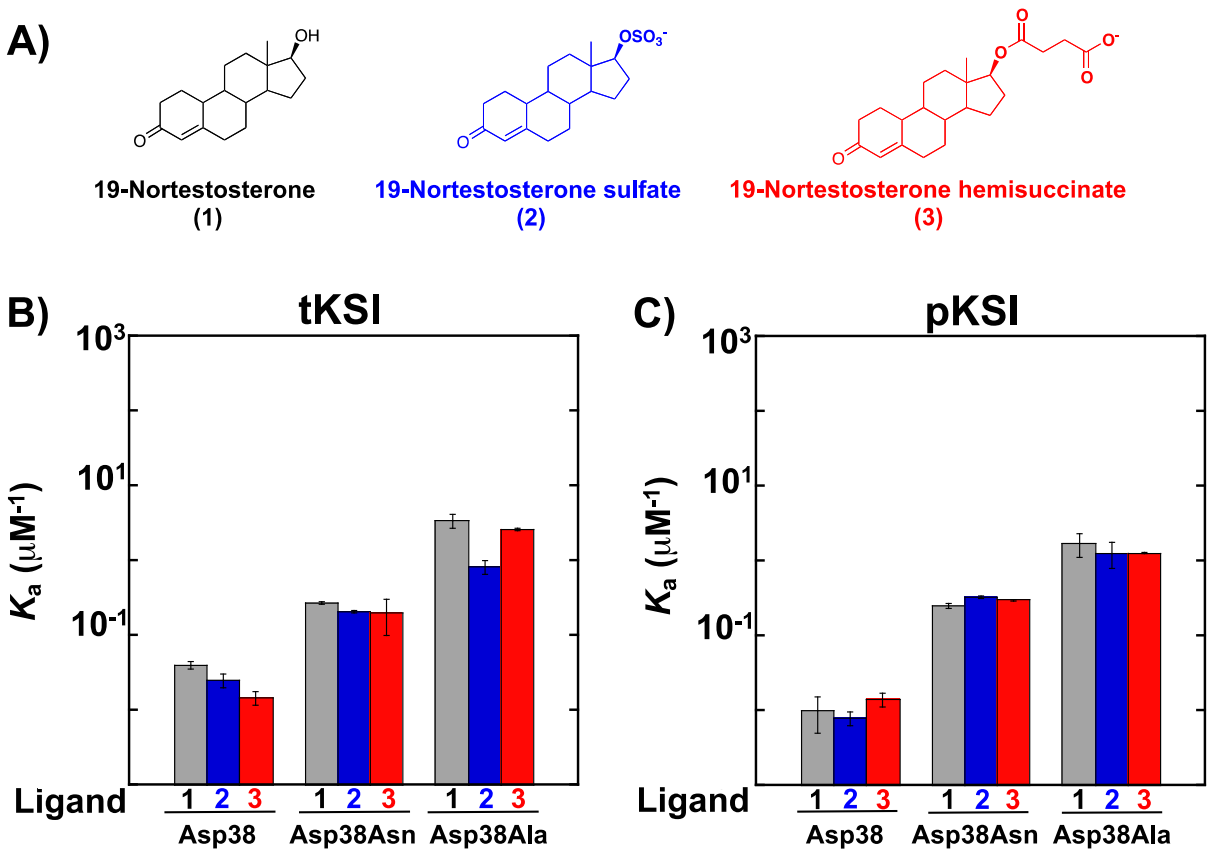

Figure S3. Binding affinity values with ligands having identical A-rings but different D-ring substituents. A) Structures of the ligands used in the measurements. B) Association constants for ligand binding to tKSI and pKSI. Ligands are color-coded and numbered as in panel A. Values are from Tables S2 and S3.

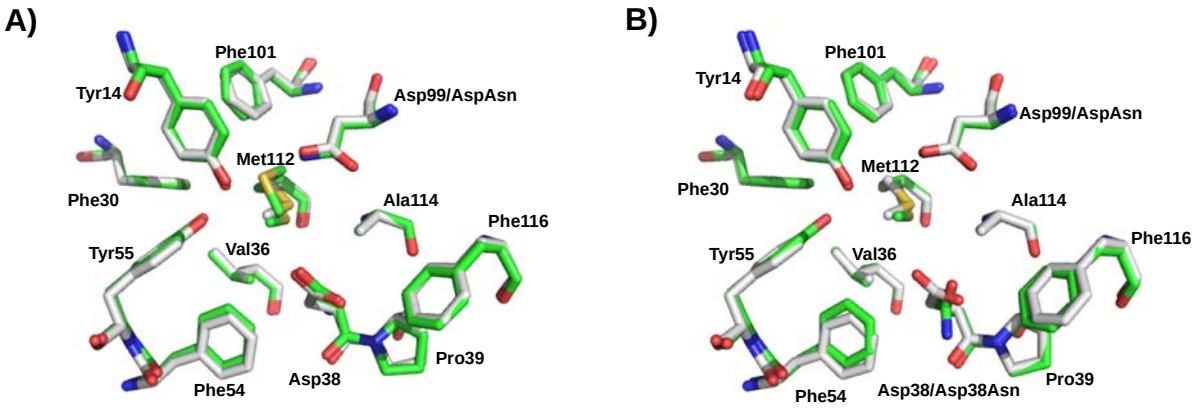

Figure S4. Superposition of the x-ray structures of the tKSI D99N and D38N/D99N mutants bound with 4AND with wild-type unliganded tKSI showing that the side chains surrounding residue 38 are superimposable. The (A) 1.6 Å D99N•4-AND structure (PDB ID 3NHX, carbon atoms colored green) and (B) 1.8 Å tKSI D38N/D99N•4AND structure (PDB ID 3NUV, carbon atoms colored green) both determined herein (see Table 2) each superimposed with the previously determined 2.3 Å wild type structure (PDB ID 8CHO, colored gray). The ligand is omitted for clarity. The overall root-mean-square deviation between the two structures for backbone atoms is 0.2 Å. X-ray data and refinement statistics are given in Table 2.

Table S1. Effects of Asp38 mutations on 19-NT association constants for tKSI and pKSI

	tKSI		pKSI	
Enzyme	K_a (μM^{-1})	K_a ratio (Mutant/WT)	K_a (μM^{-1})	K_a ratio (Mutant/WT)
Wild type	$(4.0 \pm 0.04) \times 10^{-2}$	[1]*	$(1.0 \pm 0.05) \times 10^{-2}$	[1]*
D38N	$(2.7 \pm 0.1) \times 10^{-1}$	7	$(2.5 \pm 0.2) \times 10^{-1}$	25
D38A	3.4 ± 0.7	86	1.0 ± 0.6	100

* Defined as unity for comparison

• tKSI numbering is used throughout

Table S2. Effects of Asp38 mutations on 19-NT, 19-NT sulfate, and 19-NT hemisuccinate association constants for tKSI

	19-NT		19-NT sulfate		19-NT hemisuccinate	
Residue 38	K_a (μM^{-1})	K_a ratio (Mutant/WT)	K_a (μM^{-1})	K_a ratio (Mutant/WT)	K_a (μM^{-1})	K_a ratio (Mutant/WT)
Asp	$(4.0 \pm 0.04) \times 10^{-2}$	[1]*	$(2.5 \pm 0.05) \times 10^{-2}$	[1]*	$(1.5 \pm 0.03) \times 10^{-2}$	[1]*
Asn	$(2.7 \pm 0.01) \times 10^{-1}$	7	$(2.1 \pm 0.01) \times 10^{-1}$	8	$(2.0 \pm 0.1) \times 10^{-1}$	13
Ala	3.4 ± 0.7	86	$(8.3 \pm 0.2) \times 10^{-1}$	32	2.6 ± 0.08	180

* Defined as unity for comparison

Table S3. Effects of Asp38 mutations on 19-NT, 19-NT sulfate, and 19-NT hemisuccinate association constants for pKSI

Residue 38	19-NT K_a (μM^{-1})	19-NT K_a ratio (Mutant/WT)	19-NT sulfate K_a (μM^{-1})	19-NT sulfate K_a ratio (Mutant/WT)	19-NT hemisuccinate K_a (μM^{-1})	19-NT hemisuccinate K_a ratio (Mutant/WT)
Asp	$(1.0 \pm 0.05) \times 10^{-2}$	[1]*	$(8.0 \pm 0.02) \times 10^{-3}$	[1]*	$(1.4 \pm 0.03) \times 10^{-2}$	[1]*
Asn	$(2.5 \pm 0.02) \times 10^{-1}$	25	$(3.3 \pm 0.09) \times 10^{-1}$	41	$(3.0 \pm 0.01) \times 10^{-1}$	21
Ala	1.0 ± 0.6	100	1.3 ± 0.5	160	1.3 ± 0.4	91

* Defined as unity for comparison

• tKSI numbering is used throughout

Table S4. **Residue 38 side chain•ligand distances in tKSI D38N•4AND and D38N/D99N•4AND crystal structures**

Residue 38	PDB ID	Resolution (Å)	Chain	Residue 38•ligand distance (Å)
Asp[a]	3NHX	1.6	A	2.73
Asn[b]	3NUV	1.8	A	3.16
			B	3.33
Average residue 38 side chain•ligand distance in D38N/D99N structure				3.25

[a]While KSI is a dimer, only one monomer chain is available in the D38N•4AND structure file.

[b]The electron density map showed density that allowed modeling of the ligand in a backward conformation in chain B and not in the reactive conformation in the D38N/D99N•4AND structure file.

Table S5. Effects of Asp38 mutations on 4-AND association constants for tKSI in the Pro39Ala background

Enzyme	K_a (μM^{-1})	K_a ratio (Mutant/P39A)
P39A	$(2.5 \pm 0.03) \times 10^{-2}$	[1]*
D38N/P39A	$(7.4 \pm 0.2) \times 10^{-2}$	3
D38A/P39A	$(2.1 \pm 0.2) \times 10^{-1}$	8

* Defined as unity for comparison

Table S6. Effects of Asp38 mutations on 4-AND association constants for tKSI in the Pro39Gly background

Enzyme	K_a (μM^{-1})	K_a ratio (Mutant/P39G)
P39G	$(5.5 \pm 0.1) \times 10^{-2}$	[1]*
D38N/P39G	$(4.0 \pm 0.1) \times 10^{-2}$	0.7
D38A/P39G	$(6.9 \pm 0.2) \times 10^{-2}$	1.3

* Defined as unity for comparison