Supporting Information

Evaluating the Catalytic Contribution from the Oxyanion Hole in Ketosteroid Isomerase

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Material and Methods

Materials. All reagents were of the highest purity commercially available (≥97%). All buffers were prepared with reagent grade materials or better. 5(10)Estrene-3,17-dione [5(10)EST] was from Steraloids, Inc. 5-Androstene-3, 17-dione (5-AND) was synthesized as previously described by Malhotra and Ringold and was purified by silica gel column chromatography eluting with dichloromethane as previously described by Pollack et al.[1,2] 3-Cyclohexen-1-one was synthesized as previously described.[3]

KSI Mutagenesis, Expression, and Purification. Quik-Change (Stratagene) site directed mutagenesis was used to introduce the mutations into the pKSI and tKSI genes encoded on pKK22-3 plasmids or pET21c plasmids. Mutations were confirmed by sequencing mini-prep DNA from DH5α cells. Proteins were expressed and purified as previously described.[4]

KSI Kinetics. Reactions with 5(10)EST and 5-AND were monitored continuously at 248 nm in a PerkinElmer Lambda 25 spectrophotometer. Molar absorptivities of 14,800 $M^{-1}cm^{-1}$ and 14,750 $M^{-1}cm^{-1}$ were previously experimentally determined using the

commercially available products 4-estrene-3,17-dione and 4-androstene-3,17-dione, respectively.[4,5] Reactions were conducted at 25 °C in 10 mM potassium phosphate, pH 7.2, 1 mM sodium EDTA, 2 mM DTT with 2% DMSO added as a cosolvent for substrate solubility. The values of k_{cat}, K_M, and k_{cat}/K_M were determined by fitting the initial rates as a function of substrate concentration (typically eight concentrations varied from 2 to 600 μM) to the Michaelis-Menten equation. At least three determinations at differing enzyme concentration (at least fourfold overall variation) were averaged. Reactions with 3-cyclohexen-1-one were conducted as previously described.[3]

Tests of Evaluate Stability of Oxyanion Hole Mutants. Instability of the 'carved out' oxyanion hole mutants could lead to larger observed rate reductions compared to wild-type KSI and limit determination of the effect of opening space in the oxyanion hole. The following results strongly suggest that the oxyanion hole mutants were stable and allowed accurate determination of kinetic parameters: reaction progress curves were linear during reaction timecourses for at least one hour; reactions were first-order with respect to enzyme concentration over at least a 10-fold range, as expected based on the first-order dependence of wild-type KSI with respect to enzyme concentration over at least a 10-fold range; and reaction rates determined using the same enzyme stocks on different days were identical, within experimental error.

KSI X-ray Crystallography. Single crystal diffraction data were collected at the SSRL beamline BL9-1 using a wavelength of 0.98 Å.[7] The reflections were indexed and integrated and with the programs *XDS*;[8] the intensities were scaled, merged and

converted to amplitudes with *SCALA* and *TRUNCATE*.[9] The phases were derived from the PDB entry 3CPO and refined with *REFMAC5*.[10,11] Manual model building was carried out with *COOT*.[12]

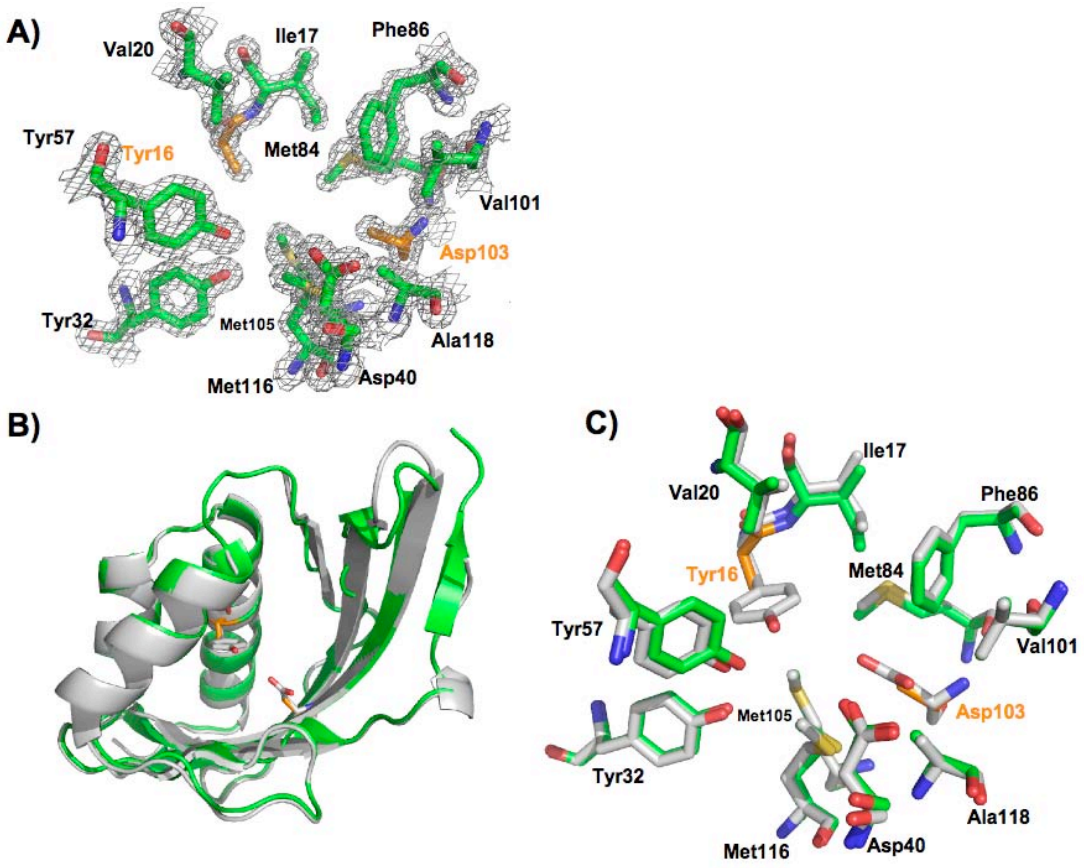

Figure S1. Overall crystal structure of pKSI Y16A/D103A is superimposable with the previously determined pKSI wild type structure. A) Sigma-A weighted $2F_0$-F_c electron density is shown for active site residues (contoured at 2.0σ). Side chains of the mutated residues are colored orange. B) Superposition of the pKSI Y16A/D103A structure determined herein (PDB ID 3T8N, carbon atoms colored green; side chains of the mutated residues are colored orange) and the previously determined 1.9 Å pKSI wild type structure (PDB ID 1OPY, carbon atoms colored white). C) Superposition of active site residues from the structures in panel B. The overall root-mean-square deviation between the two structures for backbone atoms is 0.3 Å. X-ray data and refinement statistics are listed in Table S6.

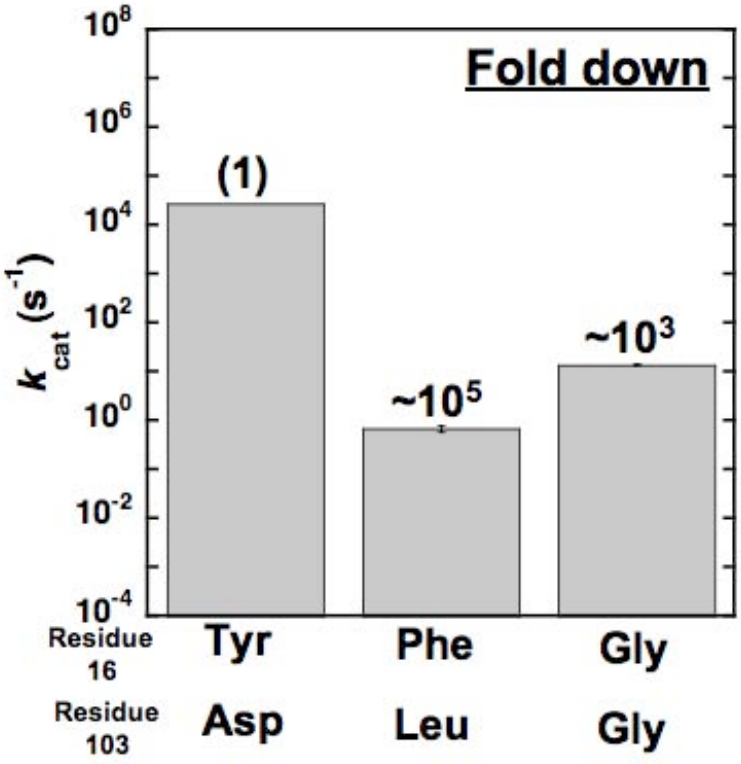

Figure S2. Effects of oxyanion hole mutations on pKSI activity (k_{cat}) using 5-AND. Values for wild type pKSI and Y16F/D103L were replotted from Choi et al.[13] Values for Y16G/D103G were determined herein and are averages and standard deviations from three or more independent measurements and are from Table S2.

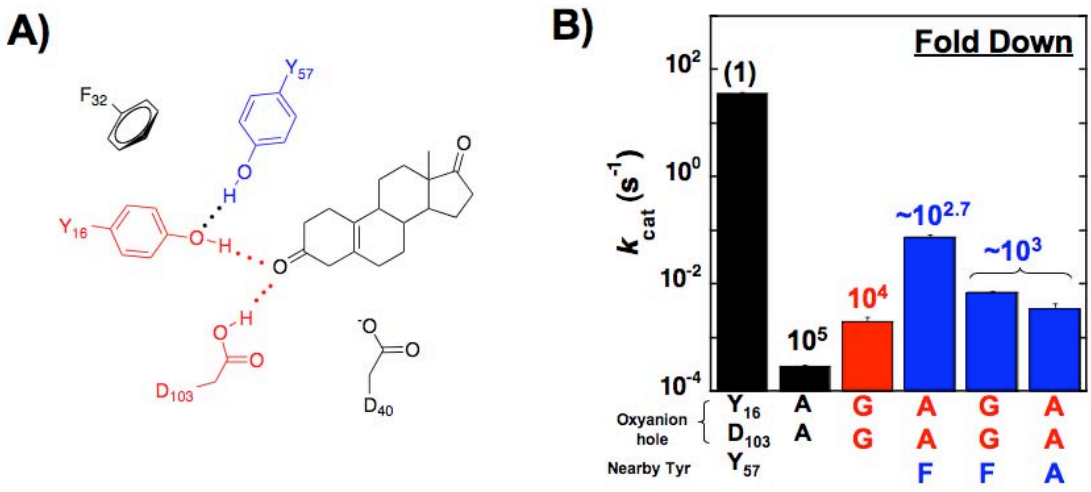

Figure S3. Effects of oxyanion hole mutations on tKSI activity (k_{cat}) using the steroid substrate 5(10)EST. A) Schematic representation of oxyanion hole hydrogen bond donors (red) a nearby tyrosine (blue). B) Rate effects from mutating the oxyanion hole hydrogen bond donors and neighboring Tyr. Values and errors are averages and standard deviations from three or more independent measurements and are from Table S3. Bars and residues are colored according to panel A.

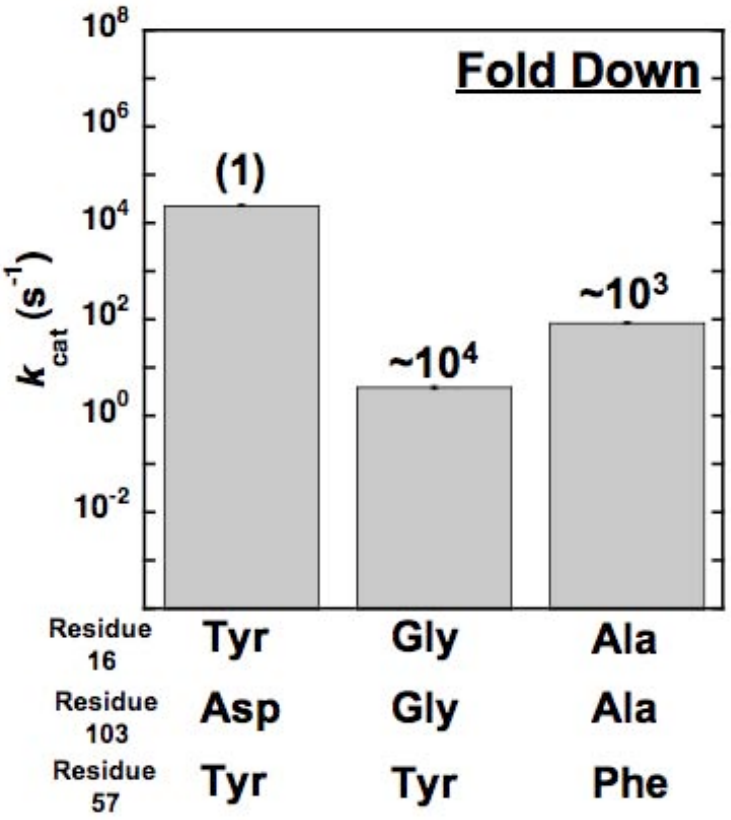

Figure S4. Effects of oxyanion hole mutations on tKSI activity (k_{cat}) using the steroid substrate 5-AND. Values and errors are averages and standard deviations from three or more independent measurements and are from Table S4.

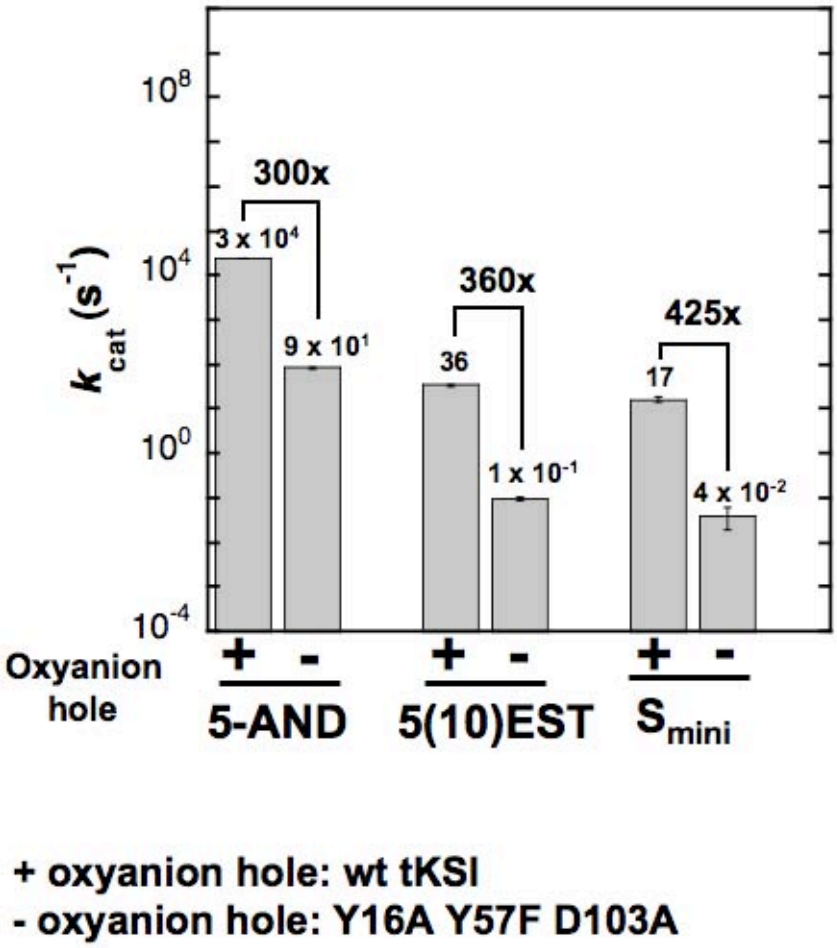

Figure S5. Effects of oxyanion hole mutations on tKSI activity (k_{cat}) using the steroid substrates 5-AND, 5(10)EST, and 3-cyclohexen-1-one (S_{mini}). Values and errors are averages and standard deviations from three or more independent measurements and are from Tables S3-S5.

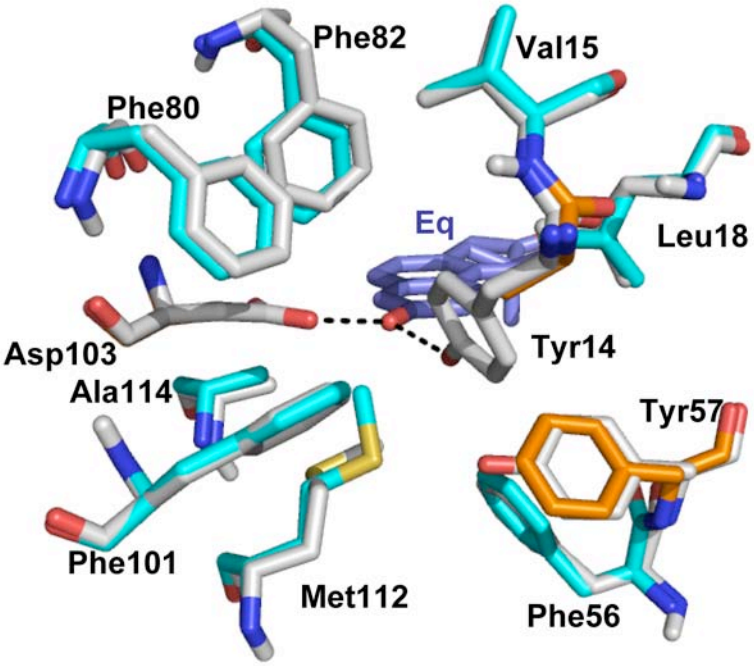

Figure S6. Superposition of the tKSI Y16A/Y57F/D103A structure determined herein (PDB ID 3T8U, carbon atoms colored cyan; side chains of the mutated residues are colored orange) and the previously determined 2.3 Å tKSI wild type structure (PDB ID 8CHO, carbon atoms colored white), and equilenin, a transition state analog, from the previously determined 2.3 Å tKSI D38N structure (PDB ID 1QJG, colored violet). X-ray data and refinement statistics are listed in Table S6.

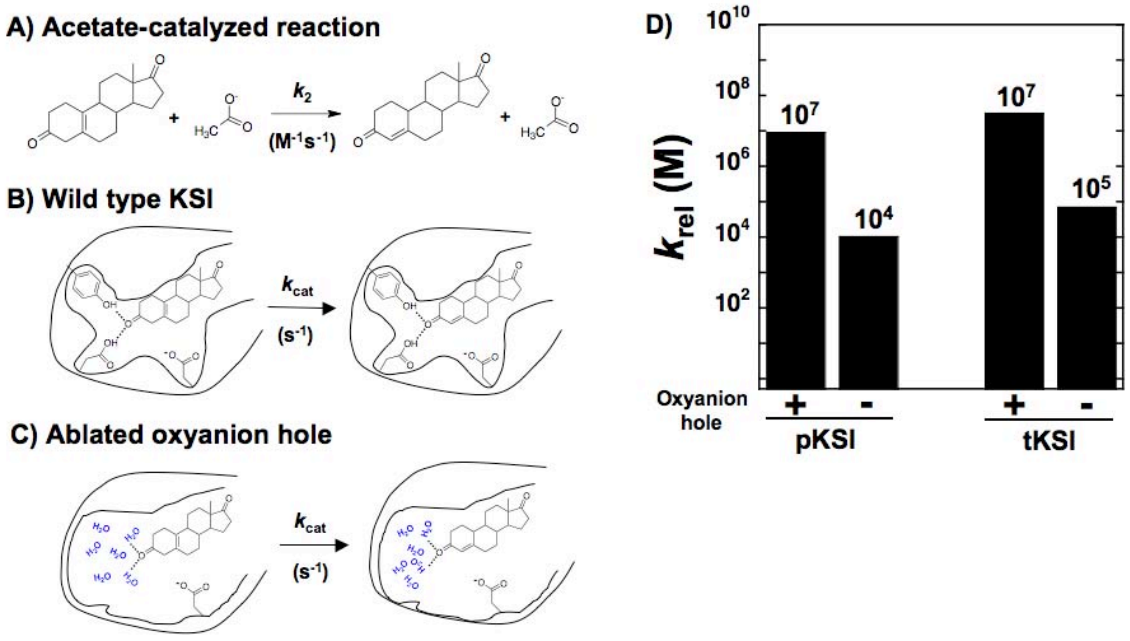

Figure S7. The catalytic contribution from KSI E•S complex relative to the acetate-catalyzed solution reaction. A) The second-order solution reaction of a KSI substrate with acetate ion. B) The first-order reaction of the wild type KSI E•S complex. C) The first-order reaction of the KSI E•S complex with less-conservative smaller oxyanion hole mutations. D) Comparison of the first-order enzymatic reaction with the second-order nonenzymatic acetate-catalyzed reaction provides a measure of the rate increase from the enzymatic E•S complexes relative to the solution reaction using the same general base functionality. The units for the ratio of the first-order KSI reaction with the second-order acetate-catalyzed reaction are molar (M).

Table S1. Effects of oxyanion hole mutations on pKSI-catalyzed isomerization of 5(10)-EST

Enzyme	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} ratio (WT/mutant)	k_{cat}/K_M ratio (WT/mutant)
Wild type	9.9 ± 0.9	30 ± 4	(3.3 ± 0.7) × 10^5	[1]*	[1]*
Y16F[a]	(5.4 ± 0.1) × 10^{-4}	30 ± 10	8 ± 1	19,000	70,000
Y16A[a]	(4.8 ± 0.4) × 10^{-2}	23 ± 2	(1.5 ± 0.3) × 10^3	200	360
Y16G[a]	(4.0 ± 0.4) × 10^{-2}	18 ± 6	(1.7 ± 0.1) × 10^3	250	310
Y16S[a]	(3.8 ± 0.5) × 10^{-2}	19 ± 3	(2.2 ± 0.4) × 10^3	260	240
Y16T[a]	(5.2 ± 0.3) × 10^{-2}	41 ± 6	(2.7 ± 0.2) x 10^3	190	200
D103L	(8.2 ± 0.6) × 10^{-2}	46 ± 3	(1.8 ± 0.2) × 10^3	120	180
D103A	(5.0 ± 0.8) × 10^{-2}	30 ± 5	(1.5 ± 0.7) × 10^3	198	220
D103G	(3.6 ± 0.5) × 10^{-1}	51 ± 5	(7.4 ± 0.9) × 10^3	28	45
Y16F/D103L	(5.1 ± 0.1) × 10^{-4}	37 ± 7	(1.4 ± 0.2) × 10^1	24,000	24,000
Y16A/D103A	(2.2 ± 0.5) × 10^{-4}	50 ± 10	3.8 ± 1	45,000	87,000
Y16G/D103G	(8.0 ± 0.3) × 10^{-3}	47 ± 7	(1.7 ± 0.2) × 10^2	1200	1900
Y16G/Y57A/ D103G	(4.6 ± 0.1) × 10^{-3}	44 ± 6	(9.1 ± 0.2) × 10^1	2100	3600
Y16G/Y32F/Y57A/ D103G	(1.2 ± 0.5) × 10^{-2}	48 ± 9	(2.8 ± 0.2) × 10^2	800	1200
Y16G/I28G/Y32F/ Y57A/D103G/ M116G/A118G	(2.1 ± 0.8) × 10^{-2}	35 ± 7	(8.5 ± 0.4) × 10^2	500	400
Y16G/I28G/Y32F/ Y57A/F86A/ D103G/M105A/ M116G/A118G	(4.2 ± 0.3) × 10^{-3}	42 ± 7	(9.9 ± 0.2) × 10^1	2300	3300

[a] Values from Kraut et al.[4]

* Defined as unity for comparison

Standard deviations from at least three experiments at different enzyme concentrations are given in parentheses

Table S2. Effects of oxyanion hole mutations on pKSI-catalyzed isomerization of 5-AND

Enzyme	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} ratio (WT/mutant)	k_{cat}/K_M ratio (WT/mutant)
Wild type[a]	2.8×10^4	50	5.5×10^8	[1]*	[1]*
Y16F/D103L[a]	$(6.7 \pm 0.1) \times 10^{-1}$	98 ± 7	6.8×10^3	42,000	81,000
Y16G/D103G	$(1.4 \pm 0.4) \times 10^1$	80 ± 2	$(1.8 \pm 0.3) \times 10^5$	2000	3000

[a] Values from Choi et al.[13]

* Defined as unity for comparison

Standard deviations from at least three experiments at different enzyme concentrations are given in parentheses

Table S3. Effects of oxyanion hole mutations on tKSI-catalyzed isomerization of 5(10)-EST

Enzyme	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} ratio (WT/mutant)	k_{cat}/K_M ratio (WT/mutant)
Wild type	36 ± 2	45 ± 4	(6.3 ± 0.2) × 10^5	[1]*	[1]*
Y16A/D103A	(3.0 ± 0.1) × 10^{-4}	20 ± 5	(1.5 ± 0.4) × 10^1	120,000	42,000
Y16G/D103G	(2.0 ± 0.4) × 10^{-3}	42 ± 5	(4.8 ± 0.1) × 10^1	18,000	13,100
Y16A/Y57F/D103A	(7.6 ± 0.5) × 10^{-2}	55 ± 3	(1.3 ± 0.2) × 10^3	470	480
Y16G/Y57F/D103G	(7.0 ± 0.1) × 10^{-3}	49 ± 6	(1.4 ± 0.2) × 10^2	5100	4500
Y16A/Y57A/D103A	(3.4 ± 0.1) × 10^{-2}	51 ± 7	(6.6 ± 0.3) × 10^2	1100	950

* Defined as unity for comparison

Standard deviations from at least three experiments at different enzyme concentrations are given in parentheses

pKSI numbering is used throughout

Table S4. Effects of oxyanion hole mutations on tKSI-catalyzed isomerization of 5-AND

Enzyme	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} ratio (WT/mutant)	k_{cat}/K_M ratio (WT/mutant)
Wild type	$(2.4 \pm 0.1) \times 10^4$	160 ± 33	$(1.5 \pm 0.1) \times 10^8$	[1]*	[1]*
Y16G/D103G	4.0 ± 0.2	143 ± 5	$(3.1 \pm 0.4) \times 10^4$	6000	4800
Y16A/Y57F/D103A	$(9.0 \pm 0.4) \times 10^1$	190 ± 5	$(4.6 \pm 0.1) \times 10^5$	300	400

* Defined as unity for comparison

Standard deviations from at least three experiments at different enzyme concentrations are given in parentheses

pKSI numbering is used throughout

Table S5. Effects of oxyanion hole mutations on tKSI-catalyzed isomerization of 3-cyclo-hexen-1-one (S_{mini})

Enzyme	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} ratio (WT/mutant)	k_{cat}/K_M ratio (WT/mutant)
Wild type	$(1.7 \pm 0.2) \times 10^{1}$	278 ± 30	$(6.2 \pm 0.1) \times 10^{1}$	[1]*	[1]*
Y16A/Y57F/D103A	$(4.0 \pm 0.2) \times 10^{-2}$	360 ± 21	$(1.6 \pm 0.6) \times 10^{-1}$	425	400

* Defined as unity for comparison

Standard deviations from at least three experiments at different enzyme concentrations are given in parentheses

pKSI numbering is used throughout

Table S6. Crystallographic Data-Collection and Refinement Statistics		
	All Data (Outer shell)	
Data Set	**pKSI Y16A/D103A**	**tKSI Y16A/Y57A/D103A**
PDB ID	3T8N	3T8U
Resolution Range (Å)	38.3-1.47 (1.50-1.47)	35.1-2.50 (2.57-2.50)
Space Group	C121	$P6_122$
a, Å	127.5	64.2
b, Å	76.7	64.2
c, Å	53.1	497.0
α, °	90.0	90.0
β, °	64.0	90.0
γ, °	90.0	120.0
Number Unique Reflections	77108 (10103)	22525 (30061)
Completeness	98.0 (88.4)	99.5 (97.0)
Multiplicity	3.6 (3.2)	10.5 (6.8)
R_{merge}, %	3.8 (87.2)	7.7 [111.6 (because of the multiplicity)]
I/σ	18.0 (1.3)	21.9 (1.6)
Refinement Statistics		
No. Residues	130	123
No. Waters	214	88
R_{work}, %	17.7 (39.1)	23.9 (34.5)
R_{free}, %	21.8 (40.3)	30.9 (44.6)
rmsd bond, Å	0.016	0.012
rmsd angle, °	1.521	1.33

$R_{merge}=\Sigma_{hkl}\Sigma_i|I(hkl)_i-\{I(hkl)\}|/\Sigma_{hkl}\Sigma_i I(hkl)_i$

$R_{work}=\Sigma_{hkl}|F(hkl)_o-\{F(hkl)_c\}|/\Sigma_{hkl}F(hkl)_o$

R_{free} was calculated exactly as R_{work} where $F(hkl)_o$ were taken from 10% of the data not included

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