Determining the catalytic role of remote substrate binding interactions in ketosteroid isomerase

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A fundamental difference between enzymes and small chemical catalysts is the ability of enzymes to use binding interactions with nonreactive portions of substrates to accelerate chemical reactions. Remote binding interactions can localize substrates to the active site, position substrates relative to enzymatic functional groups and other substrates, trigger conformational changes, induce local destabilization, and modulate an active site environment by solvent exclusion. We investigated the role of remote substrate binding interactions in the reaction catalyzed by the enzyme ketosteroid isomerase (KSI), which catalyzes a double bond migration of steroid substrates through a dienolate intermediate that is stabilized in an oxyanion hole. Comparison of a single-ring and multiple-ring substrate allowed the catalytic contribution of binding interactions with the distal substrate rings to be determined. The value of k_{cat}/K_M for a single-ring substrate is reduced 27,000-fold relative to a multiple-ring steroid substrate, suggesting that remote binding interactions with the steroid substrate contribute substantially to the KSI reaction. Nevertheless, the reaction rates for KSI-bound single- and multiple-ring substrates (k_{cat}) are within 2-fold. Further, oxyanion hole mutations have the same effect on reactions of the single- and multiple-ring substrates. These results suggest that remote binding interactions contribute >5 kcal/mol to catalysis by KSI but that local rather than remote interactions dictate the catalytic contributions from KSI's general base and oxyanion hole.

intrinsic binding energy | enzymatic catalysis | 3-cyclohexen-1-one

Over the past decades, visualization of enzyme three-dimensional structures has revealed that active sites are located in crevices or pockets. Within these active sites are found coenzymes, cofactors, metal ions, and side chains that participate in a rich array of chemical reactions. Based on this information and decades of enzymology and bioorganic chemistry, reasonable chemical mechanisms using these groups can be posited for most enzymatic reactions. Nevertheless, we still cannot quantitatively account for the enormous enzymatic rate enhancements and exquisite specificities observed by enzymes.

Over the past century, Polanyi, Haldane, Pauling, and Jencks recognized a key distinguishing feature between chemical reactions taking place in an enzyme's active site and the corresponding reaction taking place in solution (1–4). Even if the enzymatic and solution reactions use the same cofactors, metal ions, and functional groups, an enzyme can use noncovalent interactions between the enzyme and substrate to accelerate the reaction. Indeed, binding interactions between enzymes and substrates, both directly at the site of chemical transformation and with nonreacting portions of the substrate have been shown to contribute to catalysis (e.g., refs. 5–19). The mechanisms by which these noncovalent interactions can assist catalysis have been widely discussed, as briefly described in the following two paragraphs.

In the simplest scenario an enzyme can use binding interactions to localize the substrate to the active site. Beyond simple localization, these binding interactions can correctly position the reactive portion of a substrate relative to active site functional groups and relative to other substrates (7, 20–25). Concomitant with substrate binding solvent is displaced and excluded from the active site and solvent exclusion may be important in shaping the electrostatic environment within the active site (26, 27). Indeed, solvent exclusion by substrate binding has been suggested to be important for catalysis in numerous enzymes (e.g., refs. 26–32).

Jencks and others realized that remote binding interactions can do more than provide for tight binding between substrate and enzyme. Reactions of bound substrates can be facilitated by use of so-called "intrinsic binding energy", which can pay for substrate desolvation, distortion, electrostatic destabilization, and entropy loss (3, 7, 33, 34). The term intrinsic binding energy is not a molecular explanation for catalysis, but rather provides a conceptual framework for analyzing the energetics of enzymatic catalysis. In this scenario the maximum binding energy is not realized in the ground state, because aspects of the bound state, such as restricted positioning of substrates, are energetically unfavorable relative to the interactions and freedom of motion in aqueous solution. However, changes associated with achievement of the transition state, such as charge and geometric rearrangements and the formation of partial covalent bonds between positioned substrates, allow the binding energy to be more completely realized in the transition state. Indeed, results from investigations of the role of remote binding interactions in 3-oxoacid CoA transferase, triose phosphate isomerase, orotidine 5′-monophosphate decarboxylase, and other enzymes have suggested remote binding interactions provide substantial contributions to specific transition state stabilization (e.g., refs. 5–19 and 35–37).

To investigate the catalytic role of remote substrate binding interactions and concomitant solvent exclusion in the enzyme ketosteroid isomerase (KSI), we compared the KSI-catalyzed isomerization reactions of a single-ring substrate with that for the normal multiring steroid substrate (Fig. 1).

Results and Discussion

KSI catalyzes the double bond migration of steroid substrates through a dienolate intermediate that is stabilized in an oxyanion hole. In the first step of the KSI reaction, deprotonation α to a carbonyl group by an active site aspartate (Asp-40) generates a dienolate intermediate, which accepts hydrogen bonds from Tyr-16 and protonated Asp-103 in the oxyanion hole (Fig. 2). In the second step, reprotonation of the substrate at a different position gives the product (Fig. 2) (38, 39).

KSI positions a multiple-ring substrate in a hydrophobic cleft via numerous contacts with enzyme residues remote from the oxyanion hole and the site of general acid/base catalysis (Fig. 3). In addition,

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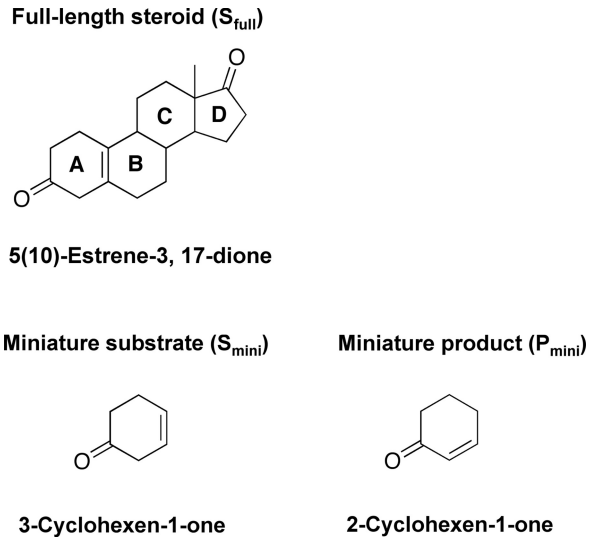

Fig. 1. Multiple- and single-ring KSI substrates and single-ring product. The single-ring substrate, 3-cyclohexen-1-one (S_{mini}), mimics the A-ring of the multiple-ring steroid substrate, 5(10)estrene-3,17-dione (S_{full}). Isomerization of S_{mini} generates the single-ring product, 2-cyclohexen-1-one (P_{mini}).

the multiple rings prevent water from approaching within ≈10 Å of the oxyanion hole in the enzyme-substrate complex (40, 41). Remote substrate binding interactions in KSI could position the substrate relative to the oxyanion hole and the general acid/base and could also influence the electrostatic environment by excluding solvent from the enzyme active site. Indeed, hydrogen bonds in a solvent-excluded oxyanion hole have been proposed to contribute to catalysis by KSI (42, 43). Conversely, solvent exclusion can also weaken hydrogen bonding (44–47).

Experimental Approach to Probe the Role of Remote-Binding Interactions in KSI. Comparison of k_{cat}/K_M values for the single-ring substrate compared with the multiple-ring steroid gives the overall catalytic contribution from remote binding interactions. However, a reduced value for k_{cat}/K_M does not indicate that binding interactions are important for specific transition state stabilization or for positioning the substrate relative to the oxyanion hole, or whether solvent exclusion by the distal rings affects the energetics of oxyanion hole hydrogen bonding. Thus, we also compared the effect of remote substrate binding interactions on reaction of the enzyme–substrate Michaelis complex (k_{cat}) for a full-length and single-ring substrate with WT KSI and a series of oxyanion hole mutants. If binding interactions with the distal steroid rings are important for specific transition state stabilization, then the simplest expectation is that k_{cat} would be reduced for the single-ring substrate. If remote binding interactions are important for positioning the steroid relative to the oxyanion hole or influence the electrostatic behavior of the oxyanion hole, removal of the oxyanion hole hydrogen bond donors by mutagenesis could have a less deleterious effect on reaction of a single-ring substrate than a steroid substrate. The experiments presented above are simple in principle, but the single-ring substrate had not previously been tested and the substrate needed to be synthesized.

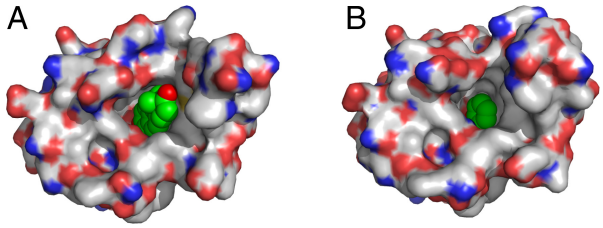

Fig. 3. Structures of KSI with bound (*A*) equilenin (1OH0) and (*B*) phenol (1PZV). The distal steroid rings are bound within a hydrophobic cavity. Surface representation generated in Pymol (66). Red is oxygen, blue is nitrogen, and white is carbon. Equilenin carbon atoms are shown in green.

Synthesis and Characterization of 3-Cyclohexen-1-One (S_{mini}). 3-Cyclohexen-1-one (S_{mini}) is a one-ring analog of the steroid substrate 5(10)estrene-3,17-dione [5(10)EST] (Fig. 1). The analog mimics the A-ring of the steroid substrate (rings labeled A–D in Fig. 1). A single ring analog of 5(10)EST was used because previous studies have shown that a chemical step is rate limiting for KSI-catalyzed 5(10)EST isomerization, unlike the more commonly used substrate 5-androstene-3,17-dione (5-AND) that is partially limited by nonchemical steps (48).

3-Cyclohexen-1-one was synthesized based on the procedure of Dzingeleski et al. (49). The final product was chromatographically purified. The isomerization product of S_{mini}, referred to herein as P_{mini}, was characterized by comparing HPLC retention times and UV absorbance spectrum to the commercially available isomerization product that was used as a standard (2-cyclohexen-1-one; Fig. S1). Treatment of S_{mini} with base generated a product identical in HPLC retention time and UV absorbance spectrum to the product standard (Fig. S2).

We then tested S_{mini} as a substrate for KSI. Incubation with KSI converted S_{mini} to a single product that was identical in HPLC retention time and UV absorbance spectrum to the commercially available product standard (Fig. S2). Product formation depended on the presence of KSI, and the reaction increased linearly with enzyme concentration and followed Michaelis–Menten kinetics, as expected for an enzymatic reaction (see below).

Differences in rate constants for KSI isomerization of the single-ring and full-length substrate could result from differences in the intrinsic chemical reactivity of the two substrates or from differential enzymatic catalysis provided by KSI. To test for differences in intrinsic reactivity, we determined the rate constants for nonenzymatic isomerization of each substrate using hydroxide ion or acetate ion as a general base catalyst. Acetate

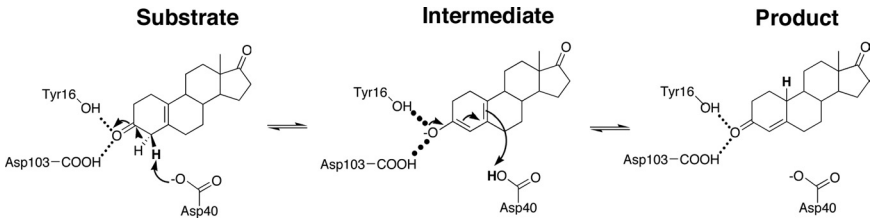

Fig. 2. Reaction mechanism for KSI catalyzed isomerization of 5(10)estrene-3,17-dione (substrate) to 4-estrene-3,17-dione (product). In the first step of the reaction, Asp-40 deprotonates the substrate to form a dienolate intermediate, which receives hydrogen bonds from oxyanion hole residues Tyr-16 and protonated Asp-103. In the second step of the reaction, the dienolate is reprotonated to form the product.

Table 1. Rate constants for isomerization reactions of S_{full} and S_{mini}

Enzyme	k_{cat}/K_M, M^{-1} s^{-1}			k_{cat}, s^{-1}		
	S_{full}	S_{mini}	Ratio (S_{full}/S_{mini})	S_{full}	S_{mini}	Ratio (S_{full}/S_{mini})
Wild type	$5.4 \pm 0.7 \times 10^5$	$2.0 \pm 0.2 \times 10^1$	27,000	9.0 ± 0.2	5.5 ± 0.5	1.6
D103N	$2.0 \pm 0.4 \times 10^4$	$4.5 \pm 0.9 \times 10^{-1}$	44,000	$2.9 \pm 0.1 \times 10^{-1}$	$2.0 \pm 0.3 \times 10^{-1}$	1.5
D103L	$1.8 \pm 0.2 \times 10^3$	$1.2 \pm 0.1 \times 10^{-1}$	15,000	$3.9 \pm 0.3 \times 10^{-2}$	$1.5 \pm 0.2 \times 10^{-2}$	2.6
Y16S	$1.9 \pm 0.7 \times 10^3$	$9.2 \pm 0.1 \times 10^{-2}$	21,000	$3.9 \pm 0.2 \times 10^{-2}$	$2.3 \pm 1.0 \times 10^{-2}$	1.7
Y16F	$8.0 \pm 1.0 \times 10^0$	$6.8 \pm 0.3 \times 10^{-4}$	12,000	$5.4 \pm 0.2 \times 10^{-4}$	$3.4 \pm 0.3 \times 10^{-4}$	1.6

mimics aspartate, the general base in the KSI active site. The nonenzymatic rate constants for isomerization of S_{full} and S_{mini} were nearly identical under all conditions [k_{HO^-} values of $(1.7 \pm 0.01) \times 10^{-1}$ M^{-1} s^{-1} and $(1.7 \pm 0.01) \times 10^{-1}$ M^{-1} s^{-1} for S_{full} and S_{mini}, respectively, and k_{AcO^-} values of $(1.1 \pm 0.1) \times 10^{-6}$ M^{-1} s^{-1} and $(6.5 \pm 1) \times 10^{-7}$ M^{-1} s^{-1} for S_{full} and S_{mini}, respectively] (Fig. S3 and Fig. S4). Thus, any difference in the enzyme catalyzed rate constants can be attributed to differences in catalysis by KSI.

Comparison of KSI Catalysis of Reactions of the Single-Ring Substrate and the Multiple-Ring Steroid Substrate. To determine the catalytic contribution in KSI from binding interactions with the distal B–D rings of a steroid substrate, we compared the k_{cat}/K_M and k_{cat} values for S_{mini} and S_{full}. Measurement of k_{cat}/K_M allows determination of the overall contribution of remote substrate binding interactions to the KSI reaction, and measurement of k_{cat} allows determination of the role of remote binding interactions to specific transition state stabilization for the two substrates starting from the enzyme–substrate complex.

As noted above, it is critical for the comparisons herein that the chemical transformation, not binding of substrate or release of product, is rate-limiting for both substrates. Results from prior studies have provided evidence that chemistry is rate-limiting for isomerization of S_{full} (50). The following results provide evidence that chemistry is also rate-limiting for reaction of S_{mini}. The values of k_{cat}/K_M for S_{mini} (and S_{full}) are much less than the diffusion controlled limit suggesting that binding is not rate-limiting for either substrate. Product release is predicted to be faster for a single-ring substrate relative to a multiple-ring steroid; as product release does not limit the reaction of S_{full} (50), it is not expected to be rate-limiting for reaction of S_{mini}. The similar effects from oxyanion hole mutations described in the next section provide additional evidence that a chemical step is rate-limiting for both substrates.

The initial rate of product formation was determined by the increase in absorbance at 240 nm and was linearly dependent on the enzyme concentration under all conditions. Initial rates were determined over a range of subsaturating and saturating substrate concentrations and fit to the Michaelis–Menten equation. The values of k_{cat}/K_M and k_{cat} for the multiple-ring steroid substrate were in good agreement with the literature values [k_{cat}/K_M values of $(3.3 \pm 0.1) \times 10^5$ M^{-1} s^{-1} and $(1.0 \pm 0.1) \times 10^5$ M^{-1} s^{-1} herein and previously reported, respectively, and k_{cat} values of 9.0 ± 0.2 s^{-1} and 9.9 s^{-1}, herein and previously reported] (Table 1) (51).

The k_{cat}/K_M value for S_{mini} of 20 ± 2 M^{-1} s^{-1} is $\approx 10^4$-fold lower than the value for S_{full}. Similarly, the binding affinity for a single-ring phenolate transition state analog is reduced $\approx 10^6$-fold relative to the binding affinity for a multiple-ringed steroid transition state analog (equilenin) (52). This large decrease in k_{cat}/K_M for S_{mini} versus S_{full} and in the binding affinity for a single-ring versus a multiple-ring transition state analog is consistent with a loss in binding interactions with the distal steroid rings (40, 41, 52). Indeed, crystal structures show a constellation of hydrophobic residues that pack around the distal steroid rings that is essentially identical for enzyme–transition state analog and enzyme–product complexes (40, 41).

To determine whether some or all of the decrease in the KSI-catalyzed reaction of S_{mini} versus S_{full} is reflected in reaction of the enzyme–substrate Michaelis complex we determined the value of k_{cat} for S_{full} and S_{mini}. The value of $k_{cat} = 5.5 \pm 0.5$ s^{-1} for S_{mini} is within 2-fold of that for S_{full}, indicating that the enzymatic reaction is not significantly compromised by removal of the B–D rings.

Effect of KSI Oxyanion Hole Mutations on Reaction of the Single- and Multiple-Ring Substrates. Versions of KSI with mutations in the oxyanion hole provide an independent test of the full use of the catalytic apparatus for reaction of S_{mini} and control for a case in which there might be, for example, compensating effects that coincidently led to identical values of k_{cat}. The single-ring substrate that lacks binding interactions to position the substrate relative to the oxyanion hole could sample a wider variety of reactive conformations that do not position or optimize electrostatic interactions with the oxyanion hole hydrogen bonding groups, relative to a well positioned, full-length substrate. If this were the case, the single-ring substrate could then react from a variety of conformers (see ref. 53). In this scenario, mutation of the oxyanion hole hydrogen bonding groups is predicted to have smaller effects on k_{cat} for reaction with S_{mini} than for reaction with S_{full}.

To test this possibility, we determined k_{cat}/K_M and k_{cat} for S_{full} and S_{mini} for a series of oxyanion hole mutants of KSI. The mutations reduced k_{cat}/K_M and k_{cat} by up to $\approx$70,000-fold and by 17,000-fold, respectively, for S_{full} and gave values in agreement with those from the literature (Table 1) (51). For each oxyanion hole mutant, the reductions in k_{cat}/K_M and k_{cat} for reaction with S_{mini} were within 3-fold of the respective decrease in k_{cat}/K_M or k_{cat} for reaction with S_{full} (Table 1). These results support the above conclusion that binding interactions with the distal steroid rings contribute to overall KSI catalysis. Nevertheless, a single-ring substrate lacking distal binding interactions is able to fully exploit the catalytic contribution from KSI's oxyanion hole.

Evaluation of an Experimental Test of the Role of Electrostatic Complementarity in KSI's Oxyanion Hole. In a previous study, Kraut et al. used a series of phenolate analogs to experimentally test the role of electrostatic complementarity in KSI's oxyanion hole, and the results suggested that oxyanion hole electrostatic complementarity provides only a modest contribution to catalysis (52). Nevertheless, it was not determined whether the behavior of single-ring phenolate transition state analogs accurately reflect the change in oxyanion hole electrostatics. Indeed, recent computational results amplified this concern, suggesting a much greater electrostatic contribution to reaction of a full-length steroid than a single-ring substrate, corresponding to 2 kcal/mol and 8 kcal/mol, respectively, and the difference was ascribed to limited prearrangement of the smaller substrate within the active site (ref. 54 and fig. 8 of the same ref.). The similar catalysis of bound single- and multiple-ringed substrates stands in contrast to the 6 kcal/mol or 10^4-fold predicted

difference in catalysis and supports the use of phenolates as an incisive probe of electrostatics in the KSI oxyanion hole (52). Nevertheless, additional tests are desirable (e.g., 55) and contributions from oxyanion hole geometric changes and electrostatic interactions at the site of general base catalysis remain to be dissected (54, 56).

Conclusions and Implications

We have conducted a test of the role of remote substrate binding interactions in the reaction catalyzed by KSI. The catalytic proficiency for a single-ring substrate, S_{mini}, is reduced $>10^4$-fold relative to a multiple-ring steroid, S_{full}, suggesting that binding interactions with the distal steroid rings provides $\approx$5 kcal/mol to catalysis by KSI. Comparison of the k_{cat}/K_M for S_{mini} with the second order rate constant for reaction of this substrate with acetate ion reveals a rate enhancement of $>10^7$, even in the absence of the distal steroid rings. The deleterious effect of the oxyanion hole mutants for reaction of S_{mini} suggest that at least some of this catalysis arises from oxyanion hole hydrogen bonds, and we speculate that positioning and/or ground state destabilization of the active site Asp general base may provide an additional rate advantage for the enzymatic reaction.

The nearly identical transition state stabilization for reaction of the enzyme−substrate complex for the single- and multiple-ring substrates suggests that local interactions position the substrate within the oxyanion hole and binding interactions and solvent exclusion by the distal steroid rings contribute little to determining oxyanion hole energetics, in contrast to prior suggestions (42, 43, 54). These results are consistent with a direct measure of active site electrostatics via a thiocyanate vibrational probe incorporated into the active-site, the exhibited the same stretching frequencies with bound single- or multiple-ringed analogs (55).

Although more study is needed, the emerging model for KSI catalysis mirrors that for other enzymes in which modest contributions from multiple catalytic strategies allow the enormous observed rate accelerations by enzymes (e.g., 57–60). For KSI, interactions with the remote steroid rings help localize the substrate to the active site and interactions with the proximal rings position the substrate with respect to the active site general base and oxyanion hole hydrogen bonds. These hydrogen bonds appear to be geometrically optimized for transition state interactions, and there may also be a modest contribution from preferential electrostatic interactions within the oxyanion hole relative to aqueous solution (48, 52, 56, 61, 62).

Materials and Methods

Materials. All reagents were of the highest purity commercially available (≥97%). 1,4-Cyclohexadienone was purchased from Acros Organics; other reagents and solvents were from Sigma–Aldrich and Fisher. Reactions using air-sensitive or moisture-sensitive reagents were carried out under an argon atmosphere. Merck silica gel (9385 grade, 230–400 mesh, 60 Å, Aldrich) was used for column chromatography. Silica gel on glass (Aldrich) was used for TLC and plates were visualized by staining with phosphomolybdic acid in ethanol (10% wt/vol).

KSI Expression and Purification. KSI from *Pseudomonas putida* was expressed and purified as described in ref. 52. Final purity was >99%, as estimated from a Coomassie-stained SDS/PAGE gel. Protein concentration was determined using the calculated molar extinction coefficient in 6 M guanidinium chloride (63).

Synthesis of 3-Cyclohexen-1-One (S_{mini}). 3,4-Epoxycyclohexene and 3-cyclohexenol were synthesized as described by Crandell et al. (64). 3-Cyclohexen-1-one was synthesized by modification of the procedure described Dzingeleski et al. (49) and is described in the following paragraph.

Under argon, pyridinium chlorochromate (9 g; 40 mmol) was suspended in anhydrous CH_2Cl_2 (40 mL) containing pyridine (0.8 mL; 2% vol/vol). The suspension was cooled to 0 °C, and 3-cyclohexenol (10 mL; 30 mmol) was added dropwise. The suspension was stirred at 0 °C for 2 h. Ether (20 mL) was added and the liquid was decanted. The black residue was washed with ether (3 × 20 mL). The combined ether washes were filtered through Florisil. The ether was evaporated under reduced pressure (>500 mbar; 30 °C) to give ≈10 mL of a yellow solution. Column chromatography (0–2% ethyl acetate in hexanes) gave 3-cyclohexen-1-one as a clear solution. Fractions containing the product were combined and the solvent was removed under reduced pressure to give 3-cyclohexen-1-one (6 mL; 60%) as a clear liquid. NMR spectra of the product were in agreement with the literature data (49, 65) and the product was further characterized by HPLC as described in the (*SI Text*).

Kinetic Assays of KSI-Catalyzed Reactions. Reactions with 5(10)estrene-3,17-dione (S_{full}) were monitored continuously at 248 nm in a PerkinElmer Lambda 25 spectrophotometer. A molar absorptivity for the product 4-estrene-3,17-dione (P_{full}) of 14,800 M^{-1} cm^{-1} was experimentally determined using commercially available P_{full}. Reactions were conducted at 25 °C in 40 mM potassium phosphate pH 7.2, 1 mM EDTA with 2% DMSO added as a cosolvent for substrate solubility. k_{cat}, K_M, and k_{cat}/K_M were determined by fitting the initial rate of activity as a function of substrate concentration (typically 10 concentrations varied from 2 to 800 μM) to the Michaelis-Menten equation. At least 3 determinations at differing enzyme concentration (at least 4-fold overall variation) were averaged, and the values agreed within 2-fold.

Reactions with 3-cyclohexen-1-one (S_{mini}) were monitored at 240 nm using either a continuous assay or a discontinuous quench assay. A molar absorptivity for the product 2-cyclohexen-1-one (P_{mini}) of 7,680 M^{-1} cm^{-1} was determined using commercially available P_{mini}. Reactions were conducted at 25 °C in 0.4–40 mM potassium phosphate pH 7.2; no dependence on buffer concentration over this range was seen for wild-type KSI, and lower buffer concentrations were necessary to reduce buffer-catalyzed background reaction in the low activity Y16F mutant. It was also necessary to use a quench assay for long time courses. Reactions were quenched at various time points by 10–1,000× dilution in 6M guanidinium chloride, 50 mM Tris pH 7.5, and then the absorbance was read at 240 nm. Control experiments with wild-type KSI demonstrated that quenching occurred within the mixing time, and rates measured for wild-type KSI by the continuous or quench assays agreed within 10%. k_{cat}, K_M, and k_{cat}/K_M were determined by fitting the initial rate of activity as a function of substrate concentration (typically 10 concentrations varied from 0.02 to 800 mM) to the Michaelis-Menten equation. At least three determinations at difference enzyme concentrations (at least 4-fold overall variation) were averaged, and the values agreed within 2-fold.

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