Published in final edited form as:
*ACS Chem Biol*. 2009 April 17; 4(4): 269–273. doi:10.1021/cb900016q.

# Evaluating the potential for halogen bonding in ketosteroid isomerase's oxyanion hole using unnatural amino acid mutagenesis

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## Abstract

There has recently been an increasing interest in controlling macromolecular conformations and interactions through halogen bonding. Halogen bonds are favorable electrostatic interactions between polarized, electropositive chlorine, bromine or iodine atoms and electronegative atoms such as oxygen or nitrogen. These interactions have been likened to hydrogen bonds both in terms of their favored acceptor molecules, their geometries, and their energetics. We asked whether a halogen bond could replace a hydrogen bond in the oxyanion hole of ketosteroid isomerase, using semi-synthetic enzyme containing *para*-halogenated phenylalanine derivatives to replace the tyrosine hydrogen bond donor. Formation of a halogen bond to the oxyanion in the transition state would be expected to rescue the effects of mutation to phenylalanine, but all of the halogenated enzymes were comparable in activity to the phenylalanine mutant. We conclude that, at least in this active site, a halogen bond cannot functionally replace a hydrogen bond.

Intermolecular forces such as hydrogen bonds, salt bridges and van der Waal's forces are critical determinants of biological structure and function. Thus, it is not surprising that intense effort has been devoted to understanding how these forces contribute to protein folding, ligand binding, and enzymatic catalysis and further in exploiting these forces to engineer biological or biomimetic recognition. Increasingly a "new" force has been added to the design toolbox – the halogen bond – that has been used much like a standard hydrogen bond for both supramolecular assembly and biological design (1–5).

A halogen bond is a principally electrostatic interaction between a classic hydrogen bond acceptor, such as the electronegative 0, N or S atom, and a polarizable, partially electropositive halogen atom, Cl, Br or I. The highly electronegative nature of F renders it a poor halogen bond acceptor but enables it to receive hydrogen bonds from standard hydrogen bond donors. Halogen bonds have been identified in numerous small molecule crystal structures on the basis of short contacts, typically 10–20% less than the sum of their Van der Waal's radii [3.37 Å for Br and O] [3,6]. Halogen bonds have been suggested to have a similar geometry as hydrogen bonds, albeit with a greater propensity towards linearity, as judged by interactions in small molecule crystals. Similar energetic properties of halogen and hydrogen bonds have also been proposed [3,6,7]. For example, a recent study of a DNA with two stable conformations, one containing a hydrogen bond and the other containing instead a Br-O halogen bond, found that

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the halogen bond-containing conformation was stabilized by 2–5 kcal/mol relative to the hydrogen bond containing conformation [4]. From a design perspective, then, it seems that one may be able to readily substitute halogen for hydrogen bonds and use them in combination to increase affinity and specificity (2,5).

Although there is substantial small molecule and computational evidence that halogen bonds are stabilizing interactions [e.g., (7–9)], few experiments have assessed the energetics of halogen bonds in biological systems. Here we ask whether or to what extent a halogen bond can functionally replace a hydrogen bond in the active site of an enzyme. As our model system we chose ketosteroid isomerase from *Pseudomonas putida* (KSI), which converts 5-androstene-3,17-dione (5-AND) into 4-androstene-3,17-dione through a dienolate intermediate (and dienolate-like transition-states) that are stabilized by hydrogen bonding in an oxyanion hole composed of Tyrl6 and the protonated Aspl03 (Scheme 1) (10–12).

Mutation of the active site tyrosine to phenylalanine, which replaces a hydroxyl hydrogen bond donor with an aromatic hydrogen, has a large, detrimental effect on catalysis of ~ 1000-fold for the substrate 5-AND (13). We reasoned that if a halogen bond was roughly energetically equivalent to a hydrogen bond, replacement of the tyrosine hydroxyl with potential halogen bond donors might lead to a rescue of catalysis relative to the phenylalanine mutant. Based purely on electrostatic considerations, and assuming that minor rearrangements in the rigid active site might accommodate the halogen atoms, an electronegative fluorine atom would be no better, and possibly even worse, than the hydrogen atom of phenylalanine, while chlorine and bromine would be able to form halogen bonds to the oxyanion transition state, providing at least some of the stabilization previously provided by the native tyrosine.

Unnatural amino acids were incorporated into semi-synthetic KSI using native chemical ligation of a peptide fragment containing the first 33 amino acids of KSI to an expressed fragment containing the remainder of the protein. A D34C mutation (<2-fold effect on activity of the WT enzyme; unpublished results) was incorporated to facilitate ligation; this enzyme is hereafter referred to as WT. Semi-synthetic WT enzyme was refolded, affinity purified, verified by electrospray mass spectrometry, and tested for activity, and this was equivalent to that of recombinant enzyme (Figure 1).

To test for the ability of a halogen donor to replace the hydroxyl hydrogen bond donor, *p*-substituted phenylalanine derivatives were incorporated into the synthetic portion of the enzyme before native chemical ligation. In addition to OH (tyrosine), fluorine, chlorine and bromine were incorporated as potential halogen bond donors, and a methyl group (Me) was incorporated as a steric control. The proteins were refolded and purified, unnatural amino acid incorporation was verified using electrospray mass spectrometry, and enzymatic activity was measured as a function of 5-AND concentration and fit to the Michaelis-Menten equation. (Table 1; Figure 2). The non-WT mutations all had kinetic parameters within 7-fold ($K_{cat}$) or 3-fold ($K_{cat}/K_M$) of the Y16F mutant.

Relative to the Y16F mutation, which replaces the tyrosine hydroxyl group with a hydrogen atom incapable of donating a hydrogen bond, all of the mutations had roughly the same level of activity, an ~3–4 order of magnitude reduction in $k_{cat}/K_M$ and $k_{cat}$ from the wild-type reaction. These results suggest that, at least for the oxyanion hole of KSI, a halogen bond cannot even partially substitute for a hydrogen bond.

An alternative explanation for the failure of halogen bond donors to rescue the loss of the hydrogen bond donor would be that another energetic interaction caused by substitution with a halogen atom counterbalances the advantage provided by halogen bonding. For example, bromine, which is considered a better halogen bond donor than chlorine, is also more hydrophobic [(LogP)chlorobenzene = 2.84; (LogP)bromobenzene = 2.99 (14,15)]. An

increasingly strong halogen bond might be accompanied by an increase in the energetic penalty caused by positioning a hydrophobic group near the charge that develops on the oxyanion group in the transition state. However, methylbenzene (toluene) has a similar hydrophobicity to chlorobenzene [LogP(toluene)= 2.73] but cannot form a halogen or hydrogen bond, yet the Me-substituted enzyme has nearly identical kinetic parameters to both the Cl-substituted enzyme and the much less hydrophobic phenylalanine mutant (Table 1 & Figure 2) [LogP (benzene) = 2.13 (15)]. Thus, it is unlikely that halogen bonds contribute significant transition state stabilization.

A model for the lack of an apparent halogen bonding interaction in these mutants is that the oxyanion hole is not flexible enough to accommodate the geometries or bond-lengths required by a halogen bond. The hydrogen bond formed by the tyrosine 16 hydroxyl to a transition state analog, equilenin, is ~2.6 Å. Simply modeling a halogen atom into published structures of KSI bound to the transition state analog equilenin (16) gives O•••Cl and O•••Br distances of 2.4 and 2.3 Å, respectively (Figure 3), due to the larger radii of the halogen atoms than oxygen (O = 1.4 Å, Cl = 1.8 Å, Br = 1.95 Å) (17). The active site distance may be too short for halogen bond formation, leading to repulsion instead of electrostatic attraction; indeed the shortest O-Cl and O-Br bond lengths observed in a survey of biological halogen bonds were 2.72 and 2.87 Å (3). However, most halogen bonds observed in this series were to carbonyl oxygens, not $sp^3$-hybridized oxygen atoms. Further, a recent *ab initio* study calculated an optimal hydroxide-bromobenzene bond distance of~2.4 Å (7).

Geometric considerations might also prevent optimal halogen bonding in the KSI active site. Halogen bonds are thought to be most energetically favorable when linear, with potentially greater sensitivity to geometry than hydrogen bonds, although it has also been suggested that deviations from linearity are likely to occur in complex biological environments (3,7). As two hydrogen bonds are made to one oxygen acceptor, the resulting deviations from linearity might prevent a halogen bond from forming. The active site might rearrange subtly upon mutation to permit more favorable bond lengths or angles, but it has been suggested that the oxyanion hole region of KSI is both quite rigid and capable of enforcing sub-angstrom distance constraints, perhaps preventing such a rearrangement (19). Indeed, chemical rescue experiments in multiple systems have demonstrated that even though an interaction or reaction can occur in solution, the constraints imposed by active site geometries and electrostatic environments can cause idiosyncratic results within enzyme cavities (20–25). It is also surprising that KSI with either hydrogen or methyl substituents on the aromatic ring at residue 16 give similar kinetic effects, and this may reflect the ability of certain accommodating rearrangements to occur within the active site. Indeed, the kinetic similarity between these two enzymes, and the active site plasticity thereby suggested, implies that it is unlikely that the failure of the potential halogen-bond donors to rescue catalysis is due to simple steric repulsion.

Although a halogen bond cannot replace a hydrogen bond within the KSI oxyanion hole, there may be other systems more amenable to engineering with halogen bonds. Nonetheless, this work serves as a caution that the similarities between halogen and hydrogen bonds may not be sufficient for a simple mix-and match style engineering. Perhaps deeper understanding of the geometry and energetics of these interactions will lead to design rules for these interactions.

## METHODS

### Peptide synthesis, purification

Peptides comprising the N-terminal 33 amino acids of KSI with a C-terminal thioester for ligation, were synthesized manually on β–mercaptopropionyl-leu-PAM resin using BOC *in situ* neutralization protocols as described previously (26). Unnatural amino acids (or tyrosine) were incorporated selectively at position 16 in the sequence. Peptides were purified by HPLC

(C18 column, Vydac) using gradient elution between A (water, 0.1% TFA) and B (9:1 acetonitrile: water, 0.09% TFA). All product masses were confirmed by ESMS.

### Construction, purification of recombinant fragment containing an N-terminal cysteine

A recombinant fragment of KSI, consisting of an N-terminal cysteine for a ligation junction followed by residues 35 to 131, was constructed and purified as follows. The 34–131 fragment was PCR amplified out of the pKK223-3 plasmid containing KSI (27) using a forward primer containing a PstI site followed by a cysteine followed by the KSI sequence starting at aspartate 35 and a reverse primer containing the terminal KSI sequence, a stop codon and a HinDIII site. Following digestion with the appropriate restriction enzymes, this PCR product was cloned in-between the HinDIII and PstI sites of the pTwin1 vector (NEB), creating a chitin-binding domain-SspB intein-C34-131KSI construct.

The fusion protein was expressed in BL21(DE3) cells grown at 37 °C by induction with 0.5 mM IPTG at an OD of ~0.6 followed by 2–3 hours of further growth. Cells were resuspended in 40 mM Tris pH 8.5,1 mM EDTA, 25% sucrose (lysis buffer) and lysed by sonication or passage through a French pressure cell. Inclusion bodies containing the fusion protein were purified by solubilization of membranes by addition of 1% sodium deoxycholate, 20 mM Tris pH 8.5, 200 mM NaCl, 2 mM EGTA, followed by centrifugation at 8000 g. Inclusion bodies were washed by resuspension in 0.25% (w/v) sodium deoxycholate, 10 mM Tris pH 8.5 several times followed by 20 mM Sodium HEPES pH 8.5, 500 mM NaCl, 1 mM EDTA (Buffer Bl) to remove detergent.

Purified inclusion bodies (which were >95% fusion protein and cleavage products, as determined by SDS-PAGE) were resolubilized in Buffer Bl supplemented with 8 M Urea. After centrifugation to remove aggregated protein, the supernatant was diluted in Buffer Bl to 0.5 M Urea at 4 °C to allow refolding of the intein. After ~1 hour, aggregates were again removed by centrifugation and the supernatant was applied to a chitin column. After washing with Buffer Bl, the column was equilibrated with 20 mM Sodium HEPES pH 7.0,1 mM EDTA, 0.1 mM TCEP (Buffer B2). The intein cleavage reaction (28) was allowed to proceed overnight at room temperature on the column. Cleavage efficiency was typically ~50%. Cleavae products were eluted with Buffer B2 supplemented with 4 M urea, as no elution occurred without urea and 4 M urea gave the best recovery of the ~ 100 amino acid fragment with the least contamination by chitin-binding domain containing fragments. The elutant, typically ~40–50% C34–131 KSI by SDS-PAGE, was buffer exchanged into 50 mM ammonium bicarbonate on a HiPrep Desalting column (Amersham) and was then lyophilized.

### Native Chemical Ligation

Peptides containing unnatural amino acids and a C-terminal thioester were ligated to the N-terminal cysteine containing recombinant fragment using native chemical ligation to form a native peptide bond (26,29). The lyophilized recombinant fragment was dissolved in ligation buffer (6 M GdmCl, 200 mM sodium phosphate, 1 mM EDTA, 25 mM TCEP, pH 8.0) to close to the solubility limit (~30 mg $mL^{-1}$; ~1.4 mM fragment). Lyophilized peptide was dissolved directly in this solution (140–450 μM). Thiophenol was added to 1% (v/v) final, and the reaction was allowed to proceed for 14–16 hours at 16 °C. The ligation mixture was then refolded by 500-fold dilution into 40 mM potassium phosphate, pH 7.2,1 mM EDTA, 2 mM DTT (40 mM KPi) followed by stirring for 1 hour at 4° C. The refolded protein was then purified by deoxycholate affinity chromatography (27) as described previously for recombinant enzyme (30), followed by buffer exchange into 40 mM KPi in a Microcon 10 kDa cutoff concentrator (Millipore). Final purity was >99% on a Coomasie-stained SDS-Page gel. Electrospray mass spectrometry (positive mode) confirmed the correct molecular weight (±3 Da) for unnatural amino acid incorporation in all cases (Table 1). Protein concentration was

determined using a calculated molar extinction coefficient at 280 nm (31), with the assumption that none of the phenylalanine derivatives affected the molar absorptivity. Yields, relative to the limiting peptide fragment, ranged from 5–20%, and 0.05–0.2 mg of pure KSI were recovered from each ligation.

### Recombinant KSI mutagenesis, expression and purification

Plasmids encoding D34C (WT) and Y16F/D34C KSI were created by QuikChange (Stratagene) site-directed mutagenesis, and were expressed and purified as described previously (30).

### Kinetics

Reactions with 5-AND were monitored continuously at 248 nm in a Uvikon 9310 spectrophotometer. A molar absorptivity for the product 4-androstene-3,17 dione of 14,750 $M^{-1}cm^{-1}$ was experimentally determined using commercially available product. Reactions were conducted at 25 °C in 40 mM potassium phosphate pH 7.2,1 mM EDTA with 2% methanol 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 (2 to 75 μM) to the Michaelis-Menten equation.

## Acknowledgements

We thank J. Schwans and P. Sigala for comments on the manuscript. This work was supported by grants to DH (NSF, MCB-0641393) and PED (NIH, GM059380). DAK was a Howard Hughes Medical Institute Predoctoral Fellow.

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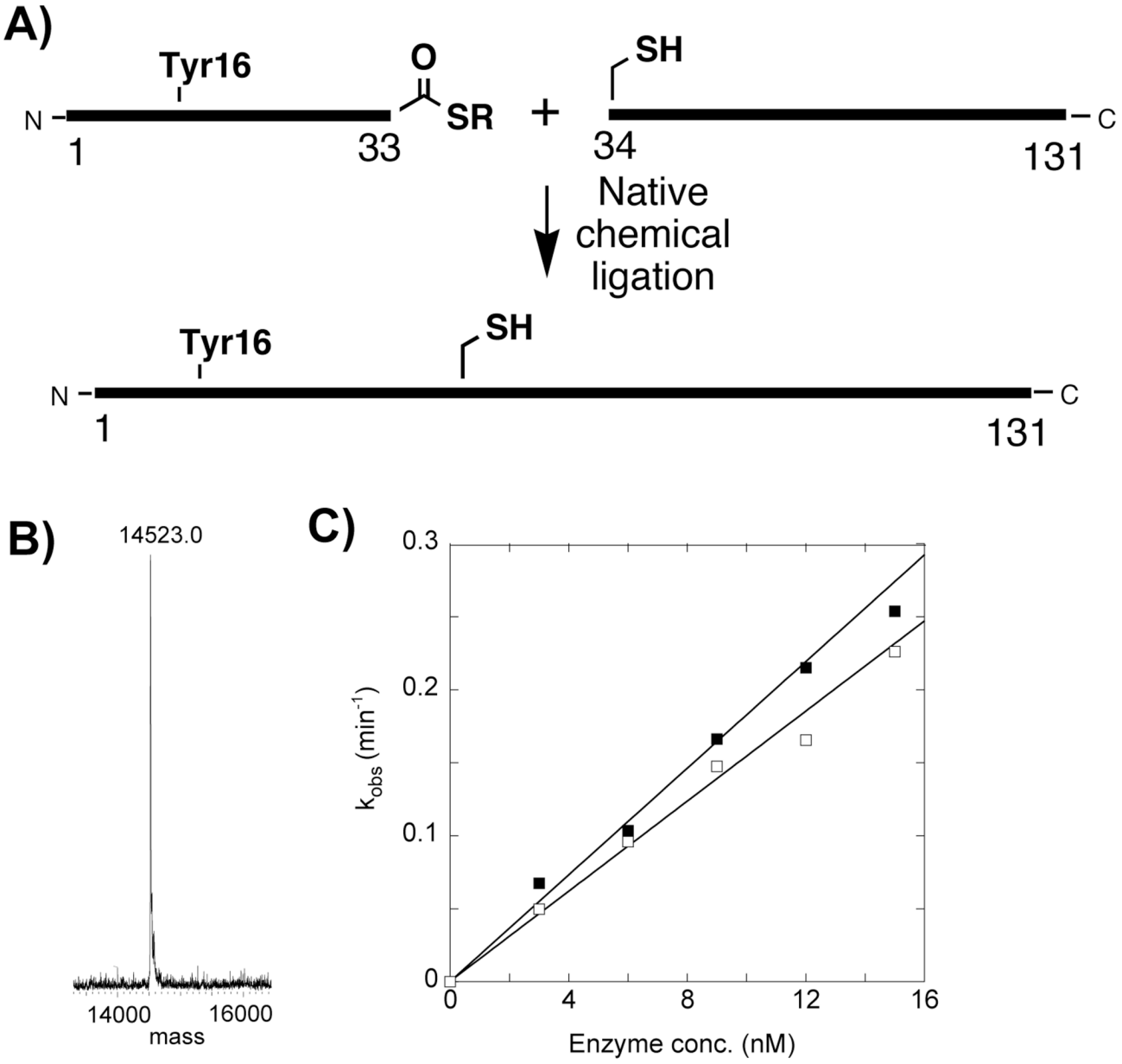



**Figure 1.**

Semi-synthesis of KSI by native chemical ligation. (**A**) A synthetic fragment (1–33) is ligated to a recombinant fragment (34–131) to generate full-length KSI. (**B**) Electrospray mass spectrum of ligated KSI (calculated mass = 14523.5). (**C**) Equivalent activity of ligated (open squares) and recombinant (closed squares) KSI using subsaturating concentrations of the slow (non-diffusionally limited) substrate 5(10)-estrene-3,17-dione. $k_{cat}/K_M = 2.6\times10^5$ and $3.0\times10^5$ $M^{-1}s^{-1}$, respectively.

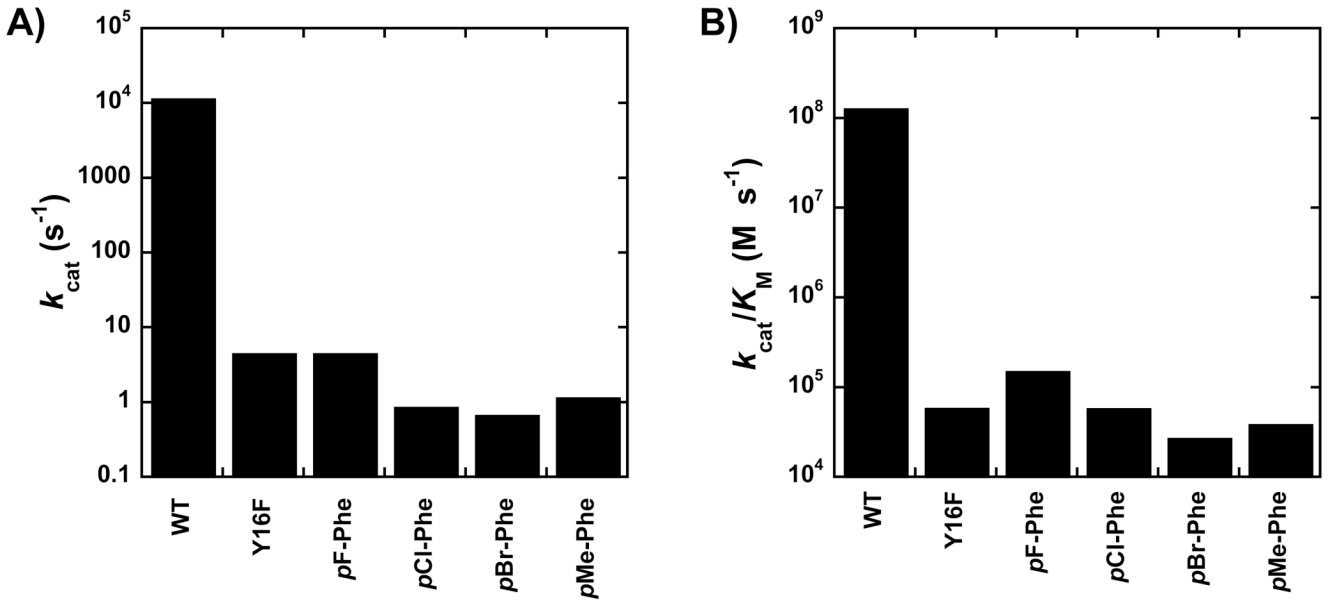



**Figure 2.**
Effects of unnatural phenylalanine variants at position 16 on (**A**) $k_{cat}$ and (**B**) $k_{cat}/K_M$ for isomerization of 5-AND.

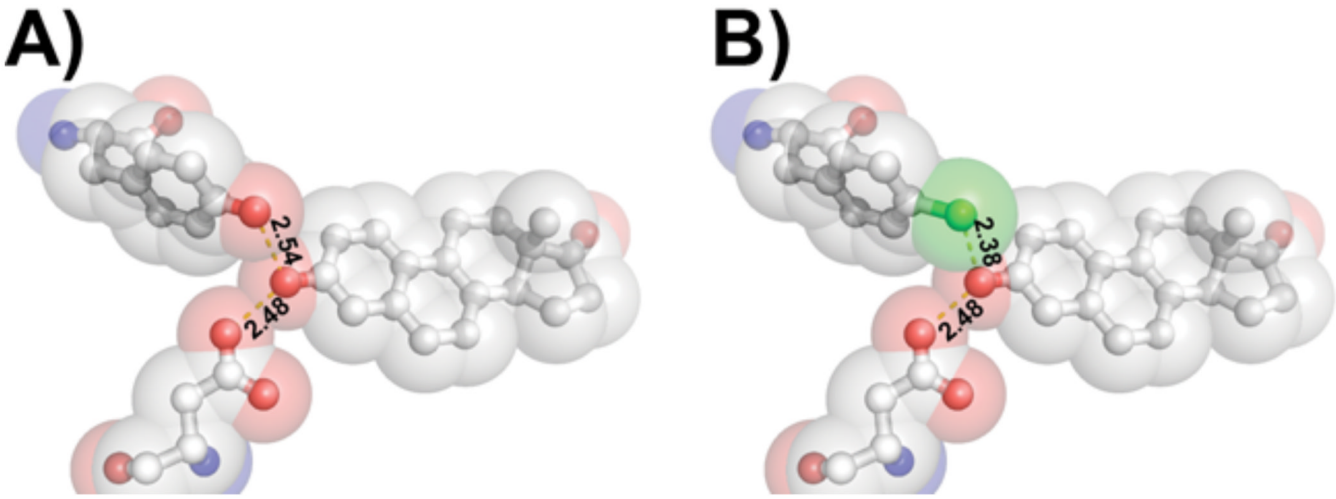
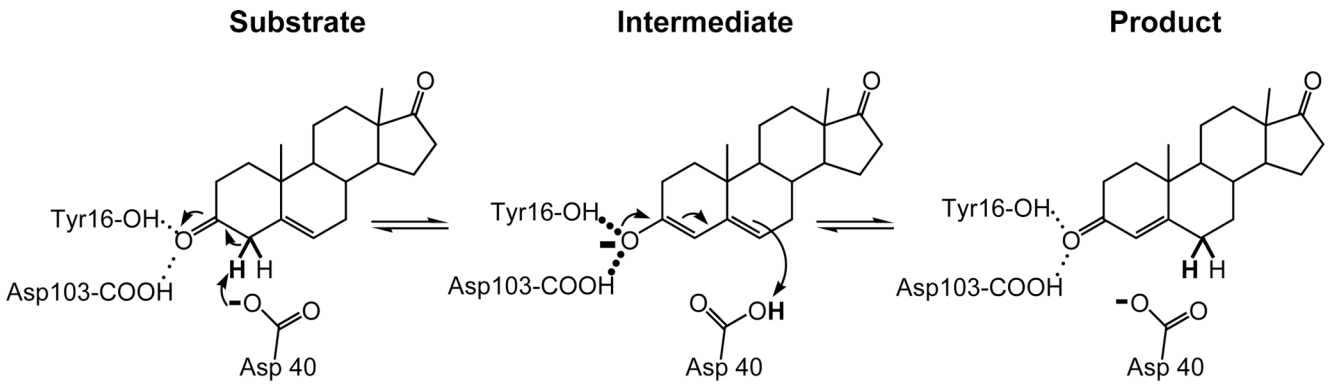



**Figure 3. Active site models**

a) Structure of WT KSI (16) bound to the transition state analog equilenin. Carbon is white, nitrogen blue, oxygen red. b) *p*Cl-Phe mutation, modeled into the same structure. Chlorine is green. Structures and modeling generated using PyMol (18).




**Scheme 1.**

**Table 1**

Effects of substituted phenylalanine mutations at position 16 on isomerization of 5-AND[a].

| Mutation at position 16 | $k_{cat}$ ($S^{-1}$) | $K_M$ (μM) | $k_{cat}/K_M$ ($M^{-1}s^{-1}$) | $k_{cat}$ effect | $k_{cat}/K_M$ effect | Mass (Da) (calc) | Mass (Da) (obs) |
|---|---|---|---|---|---|---|---|
| WT[b] | $1.2\times10^4$ | 90 | $1.3\times10^8$ | 1 | 1 | 14523.5 | 14523.0 |
| *p*F-Phe[c] | 4.7 | 30 | $1.6\times10^5$ | 2500 | 800 | 14525.5 | 14525.0 |
| *p*Cl-Phe[c] | 0.9 | 15 | $6.0\times10^4$ | 13000 | 2200 | 14542.0 | 14542.0 |
| *p*Br-Phe[c] | 0.7 | 25 | $2.8\times10^4$ | 17000 | 4600 | 14586.4 | 14585.0 |
| *p*Me-Phe[c] | 1.2 | 30 | $4.0\times10^4$ | 10000 | 3500 | 14521.5 | 14519.0 |
| Phe[d] | 4.7 | 90 | $6.1\times10^4$ | 2500 | 2100 | | |

[a] Errors are estimated to be ~20%, based on uncertainties in determining protein concentrations and experimental error.

[b] Recombinant protein gave identical results.

[c] Mutants containing unnatural amino acids are described by the substituent at the *para* position of the phenylalanine ring; *p*F-Phe contains fluorine, *p*Cl is chlorine, *p*Br is bromine, and *p*Me is methyl. WT contains a hydroxyl group at the *para* position, while Phe (Y16F) contains a hydrogen atom.

[d] RecombinantY16F protein.