Variation in relative substrate specificity of bifunctional 
β-D-xylosidase/α-L-arabinofuranosidase by single-site mutations: 
Roles of substrate distortion and recognition 

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Abstract 

To probe differential control of substrate specificities for 4-nitrophenyl-α-L-arabinofuranoside (4NPA) and 4-nitrophenyl-β-D-xylopyranoside (4NPX), residues of the glycone binding pocket of GH43 β-D-xylosidase/α-L-arabinofuranosidase from Selenomonas ruminantium were individually mutated to alanine. Although their individual substrate specificities (kcat/Km)4NPX and (kcat/Km)4NPA are lowered 330 to 280,000 fold, D14A, D127A, W73A, E186A, and H248A mutations maintain similar relative substrate specificities as wild-type enzyme. Relative substrate specificities (kcat/Km)4NPX/(kcat/Km)4NPA are lowered by R290A, F31A, and F508A mutations to 0.134, 0.407, and 4.51, respectively, from the wild type value of 12.3 with losses in (kcat/Km)4NPX and (kcat/Km)4NPA of 18 to 163000 fold. R290 and F31 reside above and below the C4 OH group of 4NPX and the C5 OH group of 4NPA, where they can serve as anchors for the two glycone moieties when their ring systems are distorted to transition-state geometries by raising the position of C1. Thus, whereas R290 and F31 provide catalytic power for hydrolysis of both substrates, the native residues are more important for 4NPX than 4NPA as the xylopyranose ring must undergo greater distortion than the arabinofuranose ring. F508 borders C4 and C5 of the two glycone moieties and can serve as a hydrophobic platform having more favorable interactions with xylose than arabinofuranose. 

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Keywords: Glycoside hydrolase; GH43; Stereoelectronic effect; Near attack conformation; Transition state 

1. Introduction 

Bifunctional enzymes displaying β-D-xylosidase (EC 3.2.1.37) and α-L-arabinofuranosidase (EC 3.2.1.55) activities are found among diverse biological species, glycoside hydrolase (GH) families (GH3, GH43, GH51, and GH54; based on protein sequence), and glycoside hydrolase clans (GH-A, GH-F and unassigned clan; based on three-dimensional structures and protein sequence relationships) in the CAZY database (Carbohydrate Active Enzymes database, http://www.cazy.org/) [1–3]. Furthermore, β-D-xylosidase/α-L-arabinofuranosidase (XA) enzymes are known to catalyze hydrolysis of glycoside bonds through either of two distinct, classical mechanisms: single displacement with inversion of anomeric stereochemistry or double displacement with retention of anomeric stereochemistry [4–11]. The common link of bifunctionality, which is not shared uniformly among XA enzymes as some have greater xylosidase activity (determined with substrate 4NPX, 4-nitrophenyl-β-D-xylopyranoside) than arabinosidase activity (determined with substrate 4NPA, 4-nitrophenyl-α-L-arabinofuranoside) and others have the opposite imbalance [8–15], owes to similarity of the glycone residues in three dimensional space such that glycosidic bonds and hydroxyl groups of β-D-xylose and α-L-arabinofuranose glycosides can be overlaid to occupy similar positions [8,11]. XA enzymes have potential utility in industrial processes where they could serve double duty in depolymerizing complex carbohydrates (i.e., arabinoxylans) of herbageous biomass to simple sugars for subsequent fermentation to fuel ethanol or other bioproducts [16–19]. In this regard, XA from Selenomonas ruminantium (SXA) of glycoside hydrolase family 43 (GH43) holds particular interest because it has been
revealed as a highly efficient catalyst for promoting hydrolysis of xylooligosaccharides [10,20].

Members of GH43, GH32, GH62, and GH68 possess 5-bladed β propeller domains. X-ray structures have been determined for GH43 XA enzymes isolated from *Bacillus subtilis*, *Bacillus halodurans*, *Clostridium acetobutylicum*, and *Geobacillus stearothermophilus*, which have 53–70% protein sequence identity with SXA [10,21]. The structures manifest a funnel-shaped active site that comprises two subsites with a single route for access by ligands. Structures of catalytically impaired mutant XAs from *G. stearothermophilus* in complex with xyllobiose are most useful for modeling ligands in the active site, particularly since there is little change in the positions of amino acid residues of subsite −1 in comparison to the structure of the unliganded wild-type XA [21] and the eight amino acid residues in the vicinity of the xylose moiety of the nonreducing end of xyllobiose, which occupy subsite −1, are fully conserved among some GH43 XA enzymes, including SXA and the XA enzymes from *B. subtilis*, *B. halodurans*, *C. acetobutylicum*, and *B. pumilus*. Substrates 4NPX and 4NPA, docked in subsite −1 are shown in Fig. 1. Notably, a hydrogen bonding network is formed among D14, D127, E186, H248, and R290 side chains; the salt bridge between D14 and R290 serves to lower the pK_a of the carboxylate group of D14, the catalytic base; and the pK_a of the carboxylate group of E186, the catalytic acid, is raised by its proximity to D127 [10,21]. Three hydroxyl groups of xyllose share H bonds with side chains of D127 and H248 (O2), D127 (O3), and D14 and R290 (O4). Because similar positions are occupied by the O2, O3, and O5 of 4NPA, similar H bonds are shared. Interactions with C2 hydroxyl groups are considered most important to catalysis in GH-mediated reactions [11,22,23].

Biochemical evidence is in accord with structural inferences that SXA (and other GH43 XA enzymes) catalyzes hydrolysis of substrates with inversion of anomeric stereochemistry, implicating a single transition state with the catalytic base (D14) serving to activate a water molecule for addition to substrate and the catalytic acid (E186) serving to protonate the leaving group; SXA catalyzes the hydrolysis of a single residue from the nonreducing end of substrate without processivity so that all products of the hydrolysis reaction are removed from the active site before initiating another catalytic cycle; mutation D127A causes the greatest erosion of catalysis among active-site residues studied;

![Fig. 1. Stereoview models of the SXA active site (subsite −1) with docked substrates 4NPX and 4NPA. Xylobiose coordinates were transferred from the X-ray structure of catalytically-impaired XA mutant (E186G) from *G. stearothermophilus* (PDB Code 2EXK) to the wild-type XA structure (PDB Code 2EXH). The eight protein residues shown (SXA numbering of residues) are fully conserved in SXA and some other GH43 XA enzymes. 4NPX and 4NPA were docked into the active site by using xylobiose as a guide. (A) 4NPX. Shown to the right: numbering system of xylose ring and distortion of xylose ring so that C1, C2, C5 and O5 are coplanar. (B) 4NPA. Shown to the right: numbering system of arabinofuranose ring and distortion of arabinofuranose ring so that C1, C2, C4 and O4 are coplanar.](image-url)
and the β-xylanase and α-arabinofuranosidase activities share the single active site of the SXA protomer [10].

The latter point was addressed with evidence from studies of catalytically impaired mutants of SXA which showed that individual mutations of the catalytic base and catalytic acid to alanine affected erosion of several thousand fold in ($k_{cat}/K_{m}$)4NPX and ($k_{cat}/K_{m}$)4NPA, but relative substrate specificities ($k_{cat}/K_{m}$)4NPX/($k_{cat}/K_{m}$)4NPA of D14A and E186A mutations remained similar to wild-type enzyme [10]. The same study exposed that, whereas $K_{m}$4NPX values were similar to wild-type SXA for D14A, D127A, H248A, and E186 mutations, the R290A mutation affected a 64-fold increase in $K_{m}$4NPX, suggesting that R290 serves as a recognition element for the substrate. It has been noted that, depending on its orientation, the C5 hydroxyl group of 4NP group has potential to conflict with the side-chains of D14, F31, and R290 [21].

In this work we set out to search for which residues, if any, of substrate –1 and in proximity to the glycone, favor the β-xylanase reaction over the α-arabinofuranosidase reaction. Off the enzyme, hydrolysis of 4NPX occurs more rapidly than 4NPP (the ΔΔG amounts to ~1.6 kcal/mol for 4NPX versus 4NPP at pH 5.3 and 25 °C; D. B. Jordan, unpublished) and this is attributable, in part, to the greater difficulty of the all-equatorial substituted [β-D-xylopyranosyl] residue of 4NPX than the α-L-arabinofuranosyl residue of 4NPA in achieving geometrical distortion to the transition state [[11,24] and references therein]. SXA-catalyzed reactions have relative substrate specificities ($k_{cat}/K_{m}$)4NPX/($k_{cat}/K_{m}$)4NPA of 12.3 and $k_{cat}$4NPX/$k_{cat}$4NPA of 11.6 (ΔΔG ~ 1.5 kcal/mol) [10]. Altogether, there is ~3 kcal/mol window available to dissect for contributions to catalysis that favor 4NPX over 4NPA.

2. Materials and methods

2.1. Materials and general methods

4NPA, 4NPX, and buffers were obtained from Sigma-Aldrich. Water was purified through a Milli-Q unit (Millipore). All other reagents were reagent grade and high purity. A Cary 50 Bio UV-Visible spectrophotometer (Varian), equipped with a thermostatted holder for cuvettes, was used for absorbance spectra and kinetic determinations. An Aviv Model 215 circular dichroism spectrophotometer (Aviv Biomedical), equipped with thermostatted cuvette holder, and a 1-mm path length quartz cuvette were used for acquiring protein spectra; 3N spectra were averaged for each protein sample. SXA was cloned from S. ruminantium, and expressed in E. coli BL21(DE3). BL21(DE3) cells containing SXA and SXA mutants were grown and the proteins were purified to homogeneity (judged by SDS-PAGE analysis) as described [10], with the addition of a final desalting, gel filtration step employing a 2.6 × 30 cm column of Bio-Gel P-6 DG desalting gel (Bio-Rad), equilibrated and developed with 20 mM sodium phosphate, pH 7.0. Mutant proteins behaved similarly to wild-type SXA in terms of yields and in E. coli (~30% of soluble protein) and elution times from chromatography columns. Circular dichroism spectra (190–260 nm, collected for samples containing 8 μM enzyme in 18 mM sodium phosphate, 4 mM Tris–HCl, pH 7.3) are characterized by a major trough at 214 nm (reflecting dominance of β structure), and the spectra are similar for wild-type SXA and mutants: mean trough values (θI) at 214 nm for three wild-type SXA samples = (–3.00 ± 0.13) × 10^6 deg·cm²·dmol⁻¹; mean trough values (θI) at 214 nm for the 13 mutant proteins = (–2.83 ± 0.14) × 10^6 deg·cm²·dmol⁻¹ with a range of (–2.52 to –3.00) × 10^6 deg·cm²·dmol⁻¹.

2.2. Preparation of mutant enzymes

Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Oligonucleotide primers are listed in Table 1. The template was pSRA1 with the SXA gene cloned into pET21 (+) [25]. Complete sequences were determined on a Model 3700 sequencer (Applied Biosystems) using 17 promoter and specific primers to confirm that only the intended mutations had been introduced. pSRA1 and its mutated plasmids were used for transformation of E. coli BL21(DE3). BL21(DE3) cells containing SXA and SXA mutants were grown and the proteins were purified to homogeneity (judged by SDS-PAGE analysis) as described [10], with the addition of a final desalting, gel filtration step employing a 2.6 × 30 cm column of Bio-Gel P-6 DG desalting gel (Bio-Rad), equilibrated and developed with 20 mM sodium phosphate, pH 7.0. Mutant proteins behaved similarly to wild-type SXA in terms of yields and in E. coli (~30% of soluble protein) and elution times from chromatography columns. Circular dichroism spectra (190–260 nm, collected for samples containing 8 μM enzyme in 18 mM sodium phosphate, 4 mM Tris–HCl, pH 7.3) are characterized by a major trough at 214 nm (reflecting dominance of β structure), and the spectra are similar for wild-type SXA and mutants: mean trough values (θI) at 214 nm for three wild-type SXA samples = (–3.00 ± 0.13) × 10^6 deg·cm²·dmol⁻¹; mean trough values (θI) at 214 nm for the 13 mutant proteins = (–2.83 ± 0.14) × 10^6 deg·cm²·dmol⁻¹ with a range of (–2.52 to –3.00) × 10^6 deg·cm²·dmol⁻¹.

2.3. Determination of steady-state kinetic parameters

Method A (discontinuous monitoring of reaction progress) was used throughout this work for determination of initial rates. Reactions (20–40 min) were initiated by adding a small aliquot of enzyme (generally 7 μl of enzyme diluted into 10 mM sodium phosphate, pH 7.0, and incubated on wet ice or at ~25 °C) to 1-ml, temperature-equilibrated (25 °C) reaction mixtures containing 100 mM sodium succinate, pH 5.3, and varied concentrations (0.2–7 mM) of 4NPX or 4NPX. Aliquots (0.02–0.2 ml) were removed from reactions at 4 to 6-min intervals, placed into cuvettes containing 0.80–0.98 ml 0.1 M NaOH for 4NPX reactions (or 1 M Na2CO3 at pH 11 for 4NPX reactions) so that the final absorbance at 400 nm and using the extinction coefficient of 18.3 cm⁻¹·M⁻¹ for 4NPX, 4NPX, and buffers were obtained from Sigma-Aldrich. Water was purified through a Milli-Q unit (Millipore). All other reagents were reagent grade and high purity. A Cary 50 Bio UV-Visible spectrophotometer (Varian), equipped with a thermostatted holder for cuvettes, was used for absorbance spectra and kinetic determinations. An AVIV Model 215 circular dichroism spectrophotometer (AVIV Biomedical), equipped with thermostatted cuvette holder, and a 1-mm path length quartz cuvette were used for acquiring protein spectra; 3N spectra were averaged for each protein sample. SXA was cloned from S. ruminantium, and expressed in Escherichia coli [25]. D14A, D127A, E186A, H248A, and R290A mutants of SXA were prepared and purified to homogeneity in the same manner as wild-type SXA [10]. Manipulations of coordinates (overlays, distance measurements, etc.) were through Swiss-PDB Viewer 3.7 (http://www.expasy.org/spdbv/) [26]. Molecular graphics images were produced using the UCSF Chimera package from the Resource of Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081) [27].

2.2. Preparation of mutant enzymes

Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Oligonucleotide primers are listed in Table 1. The template was pSRA1 with the SXA gene cloned into pET21 (+) [25]. Complete sequences were determined on a Model 3700 sequencer (Applied Biosystems) using 17 promoter and specific primers to confirm that only the intended mutations had been introduced. pSRA1 and its mutated plasmids were used for transformation

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer sequence (5’→3’)</th>
</tr>
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<tbody>
<tr>
<td>F31A</td>
<td>CTATATGTCACCCCTCCGACGTCGTTGTTGCCGAGGGACCTGGAATCAGTTAATAGG</td>
</tr>
<tr>
<td>R290H</td>
<td>GTTGTGGACGTTGCTTTTGCCTCCAGGCAAGAGCACCTACCCAA</td>
</tr>
<tr>
<td>R290K</td>
<td>GTGCTCGGGCCGGAGAAACCGCCATCCAA</td>
</tr>
<tr>
<td>R290G</td>
<td>TTGTTAGGCTGGCTTTCTGCCCGCAGGACAG</td>
</tr>
<tr>
<td>R290V</td>
<td>TGATCGGGGTTTCGAGGCCAACCGCATCAA</td>
</tr>
<tr>
<td>W73A</td>
<td>GATTCCCGCGCGCATGCCAGACACCATGACCTGCCTC</td>
</tr>
<tr>
<td>F508A</td>
<td>GCCGGCCGCCCGGATGTCGTAGCTCACCCGCGGAGCATC</td>
</tr>
</tbody>
</table>

a Underlined nucleotides indicate mutations incorporated into primers.
parameter at a single pH, $P$ is the pH-independent value of the parameter, $K_a$ is the acid dissociation constant of the group affecting concentration, $K_{a1}$ is the acid dissociation constant of the first group affecting $P$, $K_{a2}$ is the acid dissociation constant of the second group affecting $P$, $P_1$ is the limit of $P$ associated with $K_{a1}$, and $P_2$ is the limit of $P$ associated with $K_{a2}$.

$$v = \frac{k_{cat}^a S}{k_m + S}$$  \hspace{1cm} (1)

$$P = \frac{P_1}{1 + \frac{H}{K_{a1}} + \frac{H^+}{K_{a2}}}$$  \hspace{1cm} (2)

$$P = \frac{P_2}{1 + \frac{H}{K_{a2}}}$$  \hspace{1cm} (3)

$$P = \frac{P_1 + P_2 - P_1}{1 + \frac{H}{K_{a2}}}$$  \hspace{1cm} (4)

3. Results and discussion

Steady-state kinetic parameters were determined at pH 5.3 to avoid nonproductive binding of 4NPX to SXA known to occur with the native SXA at higher pH (p$K_a$ 7); such nonproductive binding does not occur with 4NPA [10]. This precaution is unnecessary for comparing relative substrate specificities ($k_{cat}/K_m$)4NPX/($k_{cat}/K_m$)4NPA because $k_{cat}/K_m$ is not affected by nonproductive binding. ($k_{cat}/K_m$)4NPX/($k_{cat}/K_m$)4NPA is nearly pH independent in the pH range 4.3–7 and above pH 7 drops by 25% to a new limit (Fig. 2). The small pH dependence of the relative parameter owes to the similar bell-shaped curves for the individual parameters ($k_{cat}/K_m$)4NPX (p$K_a$’s 5.03 and 7.21) and ($k_{cat}/K_m$)4NPA (p$K_a$’s 5.01 and 7.34). However, the pH profiles for parameters $k_{cat}$ and $1/K_m$ differ for the two substrates: the pH dependence of $k_{cat}$ describes a bell-shaped curve with an acidic limb (p$K_a$’s 3.34) and basic limb (p$K_a$ 6.98) and the pH dependence $K_{a1}$ has only the acidic limb (p$K_a$ 3.49); the pH curve of (1/$K_m$)4NPX has two acidic limbs (p$K_a$’s 4.95 and 6.71), and that of (1/$K_m$)4NPA is bell-shaped (p$K_a$’s 4.92 and 7.37). Thus, the relative parameters $k_{cat}$4NPX/$k_{cat}$4NPA and (1/$K_m$)4NPX/(1/$K_m$)4NPA drop in value at higher pH (p$K_a$ ~ 7) to endpoints of zero. Similar to the pH profile for (1/$K_m$)4NPX, the 1/$K_i$ pH profiles for inhibition of SXA-catalyzed hydrolysis of 4NPX by d-glucose, D-xylene, and L-arabinose exhibit two acidic limbs with p$K_a$’s 5 and 7 [31]. The latter, the determination that kinetic parameters of SXA acting on 4NPX and 4NPA are weakly influenced by the viscosity of reaction mixtures [10], and that some catalytically-impaired mutants exhibit similar $K_m$ values for 4NPX and 4NPA as wild-type SXA [10] suggest that $K_m$ terms reflect binding constants for the substrates. From stopped-flow studies, SXA-catalyzed hydrolysis of 4NPX and 4NPA exhibit neither bursts nor lags, suggesting that the $k_{cat}$ terms reflect the bond-breaking step of catalysis. Therefore, in addition to $k_{cat}/K_m$ values, values for $k_{cat}$ and $K_m$ are presented and discussed for the site-directed mutants of SXA.

Among the eight native residues mutated to alanine, ($k_{cat}/K_m$)4NPX/($k_{cat}/K_m$)4NPA values are similar (values range from 8.52 to 13.0) to the wild-type value of 12.3 for five mutations (D14A, W73A, D127A, E186A, and H248A), though the individual parameters ($k_{cat}/K_m$)4NPX and ($k_{cat}/K_m$)4NPA decline by greater than 99.7% in each case (Table 2). Major erosion (≥99.7%) is effected in the $k_{cat}$4NPX and $k_{cat}$4NPA terms by D14A, D127A, and E186A mutations, as (1/$K_m$)4NPX and (1/$K_m$)4NPA remain within 45% those of wild-type SXA values. For W73A, estimated values for $K_m$4NPX and $K_m$4NPA were far greater than the highest substrate concentrations used (solubility limitation of 4NPX and 4NPA; non-saturation kinetics) and consequently, $K_m$ and $k_{cat}$ values cannot be well determined; $k_{cat}/K_m$ Values are well determined, however. For H248A, $K_m$4NPX is increased by 37% and $k_{cat}$4NPX is eroded by 99.7%; $K_m$4NPA and $k_{cat}$4NPA cannot be determined owing to the high value for $K_m$4NPA. Weak binding of substrates to W73A can be reconciled from its role implicated from the X-ray structures, in forming a pocket to guide substrates to bind in the proximity of residues more directly involved in catalysis. Differential effects on binding of 4NPX and 4NPA conferred by H248A, where H248 is a central residue in the H bonding network comprising D14, D127, E186, H248, and R290, may stem from the noted propensity of 4NPX (xylose with all equatorial OH groups) to find non-productive binding modes that do not occur with 4NPA [10].
To determine kinetic parameters for wild-type and mutated SXA with substrates 4NPX and 4NPA at pH 5.3 and 25 °C, Table 2 was provided. The 96.7% decline in the relative parameter of F31A is accompanied by erosion in the individual parameters: $(k_{cat}/K_m)_{4NPX}$ (99.7%), $(k_{cat}/K_m)_{4NPA}$ (99.4%), $(1/K_m)_{4NPX}$ (45.0%), $(k_{cat}/K_m)_{4NPA}$ (89.5%), and $(1/K_m)_{4NPA}$ (97.0%) to 4NPA increased by 248%. The reduction of 99.8% in $(k_{cat}/K_m)_{4NPA}$ accounts for the drop in the relative specificity value, categorizing F31A as a relative $k_{cat}$ on $(k_{cat}/K_m)_{4NPX}/(k_{cat}/K_m)_{4NPA}$. F31 resides above the C4 OH group of 4NPX and above the C5 OH group of 4NPA (Fig. 1).

The factor of 92 drop in $(k_{cat}/K_m)_{4NPX}/(k_{cat}/K_m)_{4NPA}$ imposed by the R290A mutation is accompanied by large losses in the individual parameters: $(k_{cat}/K_m)_{4NPX}$ (16000-fold loss), $k_{cat}$ (2500-fold), $(1/K_m)_{4NPX}$ (66-fold), $(k_{cat}/K_m)_{4NPA}$ (1800-fold), $k_{4NPA}$ (180-fold), and $(1/K_m)_{4NPA}$ (10-fold). Similar contributions from drops in $k_{cat}$, $(1/K_m)_{4NPX}$ (14-fold) and $(1/K_m)_{4NPA}$ (10-fold) account for the overall drop in relative substrate specificities, categorizing R290A as a relative $k_{cat}$ and relative $K_m$ effect on $(k_{cat}/K_m)_{4NPX}/(k_{cat}/K_m)_{4NPA}$. R290 shares H bonds with and resides below the C4 OH group of 4NPX and below the C5 OH group of 4NPA (Fig. 1).

The G1196 (2.5-fold), and (1/$K_m$) increased by 1196. The progression of relative substrate specificity values approximates the physicochemical similarities of the residues R and resembles the progression for similarity to R obtained from a Point Accepted Mutation matrix (PAM250; R > K > H > Q > V > A > G [33]). Thus, it can be inferred that mutation of R290 is specific, maintaining an organized active site. Individual parameters have the progressions: $k_{cat}$, $(k_{cat}/K_m)_{4NPX}$ (wild-type R290 > R290K > Q > K > H > G > V > A > G), $(1/K_m)_{4NPX}$ (wild-type R290 > G > V > A > G), $k_{cat}$, $(k_{cat}/K_m)_{4NPA}$ (wild-type R290 > Q > K > H > G > V > A > G), $(k_{cat}/K_m)_{4NPA}$ (wild-type R290 > K > H > G > V > A > G), and $(1/K_m)_{4NPA}$ (wild-type R290 > G > V > A > G). Relative parameters have the progressions: $k_{cat}$, $(k_{cat}/K_m)_{4NPX}$ (wild-type R290 > Q > K > H > G > V > A > G) and $(1/K_m)_{4NPX}$, $(1/K_m)_{4NPA}$ (K > H > G > wild-type R290 > Q > V > A). Taken together, relative substrate specificities of 290 mutations are perturbed by combinations of relative effects on $k_{cat}$ and $K_m$.

Among the 8 subsite $−1$ residues interrogated for their contribution to catalysis by trimming their side chains to the methyl group of alanine, severity of loss in individual substrate specificity values follows the progressions: for $(k_{cat}/K_m)_{4NPX}$, D127 > R290 > E186 > D14 > W73 > H248 > F31 > F508, and for $(k_{cat}/K_m)_{4NPA}$, D127 > E186 > W73 > D14 > R290 > H248 > F508 > F31. At the top of both progressions is D127 and in the top half of both progressions are E186 and D14. Thus, the native residues are highly important to catalyzing hydrolysis of both 4NPX and 4NPA: D127, for its role in H bonding with O2 and O3 enzymes; the side chains have been termed, the “hydrophobic platform” and the interacting portion of the glycone, “the hydrophobic patch” [32].

The R290A mutation affects the greatest change (92-fold drop) in $(k_{cat}/K_m)_{4NPX}/(k_{cat}/K_m)_{4NPA}$. This mutation was followed up with mutation of R290 to additional residues resulting in the following declines in $(k_{cat}/K_m)_{4NPX}/(k_{cat}/K_m)_{4NPA}$: R290V (45-fold), R290G (12-fold), R290Q (7.2-fold), R290H (6.5-fold), and R290K (3.1-fold). Thus, the $(k_{cat}/K_m)_{4NPX}/(k_{cat}/K_m)_{4NPA}$ values for position 290 decline from wild-type in the order R > K > H > Q > G > V > A. The progression of relative substrate specificity values approximates the physicochemical similarities of the residues R and resembles the progression for similarity to R obtained from a Point Accepted Mutation matrix (PAM250; R > K > H > Q > V > A > G [33]). Thus, it can be inferred that mutation of R290 is specific, maintaining an organized active site. Individual parameters have the progressions: $k_{cat}$, $(k_{cat}/K_m)_{4NPX}$ (wild-type R290 > R290K > Q > K > H > G > V > A > G), $(1/K_m)_{4NPX}$ (wild-type R290 > G > V > A > G), $k_{cat}$, $(k_{cat}/K_m)_{4NPA}$ (wild-type R290 > Q > K > H > G > V > A > G), $(1/K_m)_{4NPA}$ (wild-type R290 > G > V > A > G). Relative parameters have the progressions: $k_{cat}$, $(k_{cat}/K_m)_{4NPX}$ (wild-type R290 > Q > K > H > G > V > A > G) and $(1/K_m)_{4NPX}$, $(1/K_m)_{4NPA}$ (K > H > G > wild-type R290 > Q > V > A). Taken together, relative substrate specificities of 290 mutations are perturbed by combinations of relative effects on $k_{cat}$ and $K_m$.

Among the 8 subsite $−1$ residues interrogated for their contribution to catalysis by trimming their side chains to the methyl group of alanine, severity of loss in individual substrate specificity values follows the progressions: for $(k_{cat}/K_m)_{4NPX}$, D127 > R290 > E186 > D14 > W73 > H248 > F31 > F508, and for $(k_{cat}/K_m)_{4NPA}$, D127 > E186 > W73 > D14 > R290 > H248 > F508 > F31. At the top of both progressions is D127 and in the top half of both progressions are E186 and D14. Thus, the native residues are highly important to catalyzing hydrolysis of both 4NPX and 4NPA: D127, for its role in H bonding with O2 and O3 enzymes; the side chains have been termed, the “hydrophobic platform” and the interacting portion of the glycone, “the hydrophobic patch” [32].

The R290A mutation affects the greatest change (92-fold drop) in $(k_{cat}/K_m)_{4NPX}/(k_{cat}/K_m)_{4NPA}$. This mutation was followed up with mutation of R290 to additional residues resulting in the following declines in $(k_{cat}/K_m)_{4NPX}/(k_{cat}/K_m)_{4NPA}$: R290V (45-fold), R290G (12-fold), R290Q (7.2-fold), R290H (6.5-fold), and R290K (3.1-fold). Thus, the $(k_{cat}/K_m)_{4NPX}/(k_{cat}/K_m)_{4NPA}$ values for position 290 decline from wild-type in the order R > K > H > Q > G > V > A. The progression of relative substrate specificity values approximates the physicochemical similarities of the residues R and resembles the progression for similarity to R obtained from a Point Accepted Mutation matrix (PAM250; R > K > H > Q > V > A > G [33]). Thus, it can be inferred that mutation of R290 is specific, maintaining an organized active site. Individual parameters have the progressions: $k_{cat}$, $(k_{cat}/K_m)_{4NPX}$ (wild-type R290 > R290K > Q > K > H > G > V > A > G), $(1/K_m)_{4NPX}$ (wild-type R290 > G > V > A > G), $k_{cat}$, $(k_{cat}/K_m)_{4NPA}$ (wild-type R290 > Q > K > H > G > V > A > G), $(1/K_m)_{4NPA}$ (wild-type R290 > G > V > A > G). Relative parameters have the progressions: $k_{cat}$, $(k_{cat}/K_m)_{4NPX}$ (wild-type R290 > Q > K > H > G > V > A > G) and $(1/K_m)_{4NPX}$, $(1/K_m)_{4NPA}$ (K > H > G > wild-type R290 > Q > V > A). Taken together, relative substrate specificities of 290 mutations are perturbed by combinations of relative effects on $k_{cat}$ and $K_m$.

Among the 8 subsite $−1$ residues interrogated for their contribution to catalysis by trimming their side chains to the methyl group of alanine, severity of loss in individual substrate specificity values follows the progressions: for $(k_{cat}/K_m)_{4NPX}$, D127 > R290 > E186 > D14 > W73 > H248 > F31 > F508, and for $(k_{cat}/K_m)_{4NPA}$, D127 > E186 > W73 > D14 > R290 > H248 > F508 > F31. At the top of both progressions is D127 and in the top half of both progressions are E186 and D14. Thus, the native residues are highly important to catalyzing hydrolysis of both 4NPX and 4NPA: D127, for its role in H bonding with O2 and O3 enzymes; the side chains have been termed, the “hydrophobic platform” and the interacting portion of the glycone, “the hydrophobic patch” [32].
of substrate and D14 and E186, for roles as catalytic base and catalytic acid. Their mutation to alanine has little effect on the relative substrate specificity. Changes in relative substrate specificities are conferred by R290A, which is among the top half of residues whose change adversely affects \( k_{\text{cat}}/K_m \) for 4NPX and in the bottom half of residues whose change adversely affects \( k_{\text{cat}}/K_m \) for 4NPA, and by F31A and F508A, which reside in the bottom half of residue changes that negatively affect \( k_{\text{cat}}/K_m \) for 4NPX and \( k_{\text{cat}}/K_m \) for 4NPA. This underscores that determinants of \( k_{\text{cat}}/K_m \) for 4NPX and \( k_{\text{cat}}/K_m \) for 4NPA are not necessarily the most important residues for maximizing individual kinetic parameters. F31, R290, and F508 represent three distinct categories of positive contributions to \( k_{\text{cat}}/K_m \) for 4NPX and \( k_{\text{cat}}/K_m \) for 4NPA of wild-type SXA: F31, relative \( k_{\text{cat}} \) effect; R290, relative \( k_{\text{cat}} \) and relative 1/\( K_m \) effects; and F508, relative 1/\( K_m \) effect. Since F508 has little influence on 4NPX while 4NPA has nearly static stereoelectronic theory, 4NPX must attain coplanarity of C1, whereas F31 and R290 residues contribute higher ratios of \( k_{\text{cat}}/K_m \) for 4NPX and 4NPA and provide differential control of 4NPX and 4NPA reactions apart from substrate binding events.

In achieving the transition-state geometry required by stereoelectronic theory, 4NPA must attain coplanarity of C1, C2, C5 and O5 in the xylose ring and 4NPA, coplanarity of C1, C2, C4 and O4 in the arabinofuranose ring. Such changes from the ground-state geometries can be met, for the most part, by raising C1 of 4NPA and 4NPX while maintaining nearly static positions of the remaining ring atoms (Fig. 1). Movement of C1 is well documented by X-ray studies of retaining GH enzymes that compare structures of Michaelis complexes with structures of trapped covalent intermediate complexes that are linked between the catalytic nucleophile and glycone C1 [11,34,35]. In the models we propose for SXA-catalyzed hydrolysis of 4NPX and 4NPA, R290 and F31 serve to stabilize the position of C4 of the 4NPX glycone (through interactions with O4) and the 4NPA glycone (through interactions with O5) so that the position of C1 can be raised for distorting the glycone rings to achieve the necessary coplanar positions. Thus, R290 and F31 could serve as anchors at the opposite end of the glycones from C1, so that as C1 migrates to its transition-state position, work is focused on distorting ring geometry rather than dispersed by raising the entire ring system or tilting the glycone (as in a seesaw). Requirements for greater movement of C1 and greater energy of ring distortion by \( \beta \)-D-xylopyranose than \( \alpha \)-L-arabinofuranose account for the more severe affects of R290A and F31A mutations on 4NPA reactions than 4NPX reactions. Upon binding substrate xylobiose, subsite –1 residues change positions very little from the unliganded state [21]; thus, SXA can be considered preformed to favor more reactive conformations of substrate, termed near attack conformations or NACs [36]. The proposed mechanism for ring distortion in the SXA reactions resembles the proposed mechanism in the pre-organized active site of scytalosine dehydratase where one end of the bicyclic substrate is anchored by H bonds between an N residue and substrate’s phenolic OH group as the opposite end of substrate is pushed downward by a highly mobile F residue and pulled downward by H bonds between substrate’s C3 OH group and a H residue [37]. The scytalosine dehydratase mechanism of substrate distortion was brought to light by comparing relative substrate specificities for substrates that differ in energies needed for ring distortion (to transition-state geometries mandated by stereoelectronic principles) harbored by site-directed mutants and wild-type enzyme. By employing a similar strategy for SXA, we believe anchoring residues of SXA (F31 and R290) have been identified; missing is identification of enzyme residue (s) of subsite +1, if any, that favor C1 migration (the force).

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