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Homologous Alkalophilic and Acidophilic L-Arabinose Isomerases Reveal Region-Specific Contributions to the pH Dependence of Activity and Stability

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Bacterial strains and culture conditions. Thermoacidophiles able to utilize L-arabinose as their sole carbon source were isolated from volcanic hot springs at Tankuban Prahu in Indonesia. The bacteria were grown on a slightly modified DSMZ 402 medium containing 3 g of L-arabinose (as the sole carbon source), 1 g yeast extract, 0.2 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaCl_2 \cdot 2H_2O$, 0.1% (v/v) trace element solution (0.1 g $ZnSO_4 \cdot 7H_2O$, 0.03 g $MnCl_2 \cdot 4H_2O$, 0.3 g H_3BO_3, 0.2 g $CoCl_2 \cdot 6H_2O$, 0.01 g $CuCl_2 \cdot 2H_2O$, 0.02 g $NiCl_2 \cdot 6H_2O$, 0.03 g $Na_2MoO_4 \cdot 2H_2O$/liter) per liter at pH 2 and 65ºC. The isolate TP-7, which showed the highest L-arabinose isomerase (AI) activity, was identified as a species of the genus *Alicyclobacillus*, based on 16S rRNA gene sequence analysis (The GenBank accession number, JX218020).

Cloning, sequencing, and expression of the *araA* gene from *Alicyclobacillus* sp. TP-7. An internal sequence of the *araA* gene encoding AI was obtained from *Alicyclobacillus* sp. TP-7 by PCR using the genomic DNA of isolate TP-7 as a template with the degenerate primers DaraAF and DaraAR, as described previously (6). The resulting amplified 770 bp internal gene was sequenced and the full length *araA* gene was obtained by inverse PCR using the sequence based primers IASAI-F (5'-CGCGTCCTGAAGGTGCTCGCCGAGGGC-3') and IASAI-R (5'-CAAAAGCGGCTTTCGCAGCGCCAACAG-3'). Genomic DNA (100 ng) was digested with *Hind* III for 2 h at 37ºC and heated at 80ºC for 20 min. The 2–4 kb DNA fragments were purified using a QIAEX II Gel Extraction Kit (Qiagen), diluted to 3 ng of DNA per µl (50 µl), and circularized with 2 U T4 DNA ligase (Promega) per µl in ligase buffer containing 1 mM ATP for 16 h at 16ºC. The components and conditions for inverse PCR were described previously (6). Inverse PCR products were purified, cloned into a pGEM-T Easy vector (Promega), and sequenced. A final PCR amplicon containing the entire *araA* coding sequence was prepared using

the forward/reverse primer pair ASAI-F (5'-CATATGAAGATGCCGGCTTATGAATTC-3', the *Nde* I site is underlined) and ASAI-R (5'-AAGCTTTCAGCGGCCGTACACCGCCTCGTT-3', the *Hind* III site is underlined). PCR was performed in 1× PCR buffer (50 μl) containing 2 mM $MgCl_2$, 20 ng DNA, 10 pmol primers, 200 μM dNTP mix, and 2.5 U Ex-tag DNA polymerase (Takara). After a 4 min initial denaturation at 94ºC, each DNA was amplified by 30 cycles consisting of a 30 sec denaturation at 94ºC, a 30 sec annealing at 60ºC, and a 1 min extension at 72ºC, followed by a final extension step of 5 min at 72 ºC. The PCR product was cloned into the pGEM-T easy vector and transformed into *E. coli* DH5α competent cells. Transformants were selected on Luria-Bertani (LB, Difco)-ampicillin (100 μg/ml) plates containing 0.01% X-Gal. Plasmid DNA was isolated from clones with inserts and digested with *Nde* I and *Hind* III. The digested DNA was purified and ligated into the pET-22b(+) vector to yield pET-ASAI, and the products were transformed into *E. coli* BL21 (DE3) competent cells. The pET-AAAI vector harboring the *araA* gene of *A. acidocaldarius* was prepared as described previously (6). For the expression of the wild-type (ASAI and AAAI) and mutant enzymes (hybrids), transformed *E. coli* BL21(DE3) cells were grown at 37°C in 1 liter of LB medium containing 100 μg ampicillin per ml, induced at the mid-exponential phase (A_{600} = 0.5~0.6) with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), grown for an additional 4 h at 37°C, and harvested by centrifugation (10,000 × g, 20 min, 4°C).

Construction of chimeric AIs.

To construct chimeric AIs, the expression vectors pET-AAAI and pET-ASAI (6) containing *A. acidocaldarius* and *Alicyclobacillus* sp. TP-7 *araA* genes, respectively, were used as templates for PCR. For convenience, the *Nde* I sequence in the internal *araA* gene encoding AAAI (6) was

modified by site-directed mutagenesis using the forward/reverse primers 5'-CTTGGCGCCCACATGCTCGAAGTATGCC-3' and 5'-GGCATACTTCGAGCATGTGGGCGCCAAG-3' (silent point mutations are underlined). For construction of hybrids I and II, the N-terminal 818 bp fragment of ASAI was amplified by PCR using the primers 5'-CATATGAAGATGCCGGCTTATGAATTC-3' (the *Nde* I site is underlined) and 5'-CGTGAAGGCCTCGAAGCC-3' (the *Stu* I site is underlined). In parallel, an 824 bp fragment from the N-terminal region of AAAI was amplified by PCR using the forward/reverse primer pair 5'- CATATGATGCTGTCATTACGTCCTTATGAA-3' (the *Nde* I site is underlined) and 5'-CGTGAAGGCCTCAAA-3' (the *Stu* I site is underlined). The amplified DNA fragments were ligated into a pGEM-T easy vector and used to transform *E. coli* DH5α competent cells. Transformants were selected on X-Gal LB plates, and plasmid DNA was isolated and digested with *Nde* I and *Stu* I. As described above, the C-terminal 670 bp and 667 bp fragments from AAAI and ASAI, respectively, were also amplified by PCR using the primers 5'-TTTGAGGCCTTCACG-3' with an introduced *Stu* I site (underlined) and AAAI-R (6), and 5'-TTCGAGGCCTTCACG-3' with an introduced *Stu* I site (underlined) and ASAI-R, respectively. Subsequently, the amplified N- and C-terminal fragments of ASAI were ligated with the C- and N-terminal fragments of AAAI at the *Stu* I site and digested with *Nde* I and *Hind* III. Those DNA fragments were then ligated into a pET-22b(+) vector digested with *Nde* I and *Hind* III to yield pPET-hybrids 1 (1485 bp) and 2 (1491 bp) (Fig. 3). In turn, the hybrids I-E267K and II-K269E were created by site-directed mutagenesis using pET-hybrids I and II as the DNA templates, respectively. The forward/reverse primer pairs (5'-TTCCTTAAGGAAGGCGGC-3' and 5'-GCCGCCTTCCTTAAGGAA-3' for I-E267K, and 5'-TTCCTGGAGGACGGGAAT-3' and 5'-ATTCCCGTCCTCCAGGAA-3' for II-K269E) were designed to introduce the corresponding

mutations into the hybrids I and II. Clones were sequenced to verify the presence of the mutation. Upon hybridization and confirmation of a single coding region mutation, the hybridized and mutated plasmids were reintroduced into *E. coli* BL21(DE3) cells and expressed as described above.

Purification of AIs

All purification steps were carried out using the ÄCTA purifier system (Pharmacia) at 4°C. To purify the recombinant wild-type AIs and their hybrids, cell pellets were resuspended in 50 ml of 20 mM Tris-HCl buffer (pH 8.0), disrupted by sonication, heated at 65°C for 15 min, and centrifuged at 20,000 × g for 30 min to remove cell debris and *E. coli* proteins. The supernatants of ASAI, AAAI, and their hybrids were filtered through a 0.2 μm filter and loaded onto Hiprep 16/10 Q-XL columns (20 ml) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The columns were washed with 20× volume of the same buffer, and a gradient of NaCl (from 0 to 500 mM) was applied at 3 ml/min. Active fractions of AIs were pooled and concentrated in 20 mM Tris-HCl buffer (pH 7.9) using a Centriprep filter (Millipore) with a 10 kDa cutoff, and the concentrates were further purified on a HiLoad 16/60 Superdex 200 gel-filtration column equilibrated with 20 mM Tris-HCl (pH 7.5) and stored at 4°C. Protein concentrations were determined by the bicinchoninic acid method (9), with bovine serum albumin as the standard. Enzyme fractions were analyzed by sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis (PAGE) and visualized with Coomassie Brilliant blue R250 (Sigma). The relative molecular masses of the purified enzymes were determined by SDS-PAGE (3).

Enzyme activity assay and kinetics. For initial screening of thermoacidophiles producing AI, cell pellets (A_{600} = 1) were resuspended to a final concentration of 1% toluene in 50 mM sodium acetate buffer (pH 5.5) and then vortexed vigorously for 2 min (6). The cell lysates were added to the reaction mixture containing 0.1 M L-arabinose, 1 mM $MnCl_2$, and 50 mM sodium acetate buffer (pH 5.5) to a final volume of 125 μl. The reaction mixtures were incubated at 70°C for 20 min. Activity assays were performed in buffers appropriate for AAAI and ASAI at 65 and 70°C, respectively, as described previously (6). The amount of L-ribulose formed was quantified by the cysteine-sulfuric acid-carbazole method with measurement at A_{560} (2). One unit of AI activity was defined as the amount of enzyme necessary to produce 1 μmol of product per min under these conditions. The kinetic parameters of AIs were determined in the same reaction mixtures as described above, except that AI was assayed over a 2 min incubation to obtain the initial reaction rate. The concentration of L-arabinose ranged from 5 to 300 mM. Kinetic data were analyzed using Lineweaver-Burk plots to determine $V_{\max}$ and K_m.

Biochemical characterization of AI. To determine the optimum pH, AI activity was measured with L-arabinose as a substrate as described above, except that the reaction buffer was replaced by 50 mM sodium acetate buffer (pH 4.5 to 6.0), 50 mM potassium phosphate buffer (pH 6.0 to 8.0), 50 mM Tris-HCl buffer (pH 7.0 to 9.0), and 50 mM Glycine-NaOH buffer (pH 9.0 to 10.5). All pH values were adjusted at room temperature, and the $\Delta pK_a/\Delta T_s$ for each buffer were taken into account in analyzing the results. In case of reaction temperature, AI activity was assayed at various temperature ranges (45 to 85°C) at each optimum pH. For pH stability studies, the enzyme was incubated at various pH values for different periods of time and residual AI activity was measured at 65°C under the assay conditions described above.

To investigate the effects of various metal ions on enzyme activity, metal ions were removed from the purified AI by treatment with 10 mM EDTA at 40°C for 1 h, followed by overnight dialysis against 50 mM Tris-HCl buffer (pH 8.5) at 4°C with several changes of buffer. The dialyzed enzyme was preincubated at room temperature for 15 min in the presence of 1 mM $CoCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $ZnCl_2 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, $FeCl_2 \cdot 6H_2O$, or $NiCl_2 \cdot 6H_2O$, and AI activity was assayed under standard conditions.

Circular dichroism. Circular dichroism (CD) measurements were carried out using a Jasco J-810 spectropolarimeter with a Peltier temperature-controlled cuvette holder. Wild-type and hybrid AIs (0.3 mg/ml) were preincubated at 25°C in 10 mM sodium acetate buffer (pH 5), 10 mM potassium phosphate buffer (pH 6 to 7), and 10 mM Tris-HCl buffer (pH 8 to 9). The CD spectra of enzyme samples in cuvettes with a 0.1 cm path length were recorded in the far-UV region (190 to 240 nm). Scans were collected five times at 0.05 nm intervals with a 1 nm bandwidth. Each spectrum was corrected by subtracting the spectrum of the solution containing the used buffer. The high α-helical content of AIs dominates the spectrum of the native protein, with a characteristic negative value of ellipticity at 222 nm, which disappears upon unfolding. Following complete denaturation in 6 M GdnHCl, a large decrease in CD intensity was observed, indicating the loss of secondary structure. Guanidine hydrochloride (GdnHCl)-induced unfolding of AI was performed isothermally in the presence of 10 mM of each buffer at pH values ranging from 5 to 9 and 20°C. Changes in ellipticity at 222 nm were monitored by CD spectropolarimetry. The data were fitted by nonlinear least-squares analysis using a linear extrapolation model. To determine the stability curve, free energies of unfolding (ΔG_U) were calculated from the GdnHCl denaturation of AIs at several pH values, assuming a two-state

model and a linear relationship between ΔG_U and [GdnHCl] (1, 5, 8). The ratio of denatured and total protein in the transition range, f_d, was evaluated from the signal ε relative to the signals of the native and unfolded baselines, according to the following equation: $f_d = (\varepsilon_N - \varepsilon)/(\varepsilon_N - \varepsilon_U)$, where ε_N and ε_U represent the CD signal of the fully folded and unfolded forms of the protein, respectively, in the transition region.

TABLE S1. Comparison of the biochemical properties of thermoalkalophilic ASAI and homologous thermophilic AIs

Property	AAAI	GSAI	ASAI
$Temp_{opt}$ (°C)	65	70	70
pH_{opt}	6–6.5	7–7.5	8.5
apparent M_r (10^3)			
Oligomeric structure	α4	α4	α4
Subunit	57 (56.04)[a]	57 (56.09)	56 (54.87)
pI	5.0	5.6	5.63[b]
Metal ion requirement for AI activity	Mn^{2+}, Co^{2+}, Mg^{2+}	Mn^{2+}, Co^{2+}, Mg^{2+}	Mn^{2+}, Mg^{2+}
Kinetic parameters			
V_{max} (μmol/mg·min)	35.5	36.5 ± 0.8[c]	49.7 ± 3.2
K_m (mM)	48	63 ± 3	52.4 ± 5.2
k_{cat}[d]/K_m ($mM^{-1}·min^{-1}$)	41.5	32.5	52.0 ± 1.4
Reference	(6)	(4)	This study

[a]Calculated molecular weights of the deduced amino acid sequences.

[b]Calculated isoelectric point (pI) of the deduced amino acid sequence.

[c]Data are the means ± SDs.

[d]The k_{cat} is the number of substrate molecules reacted per active site per min.

TABLE S2. Comparison of the deduced amino acid sequences of the wild-type and hybrid AIs.

Enzyme source	pH_{opt}	No. of basic amino acids (K, R)	No. of acidic amino acids (D, E)	Basic (K, R)/ Acidic (D, E) ratio
ASAI	8.5	53 (15, 38)	68 (28, 40)	0.779
AAAI	6.0–6.5	52 (20, 32)	69 (25, 44)	0.754
Hybrid I	7.5–8.0	54 (18, 36)	69 (28, 41)	0.783
Hybrid II	6.5–7.0	51 (17, 34)	68 (25, 43)	0.750
Hybrid I-E267K	7.0–7.5	55 (19, 36)	68 (29, 39)	0.809
Hybrid II-K269E	7.0–7.5	50 (16, 34)	69 (24, 45)	0.725

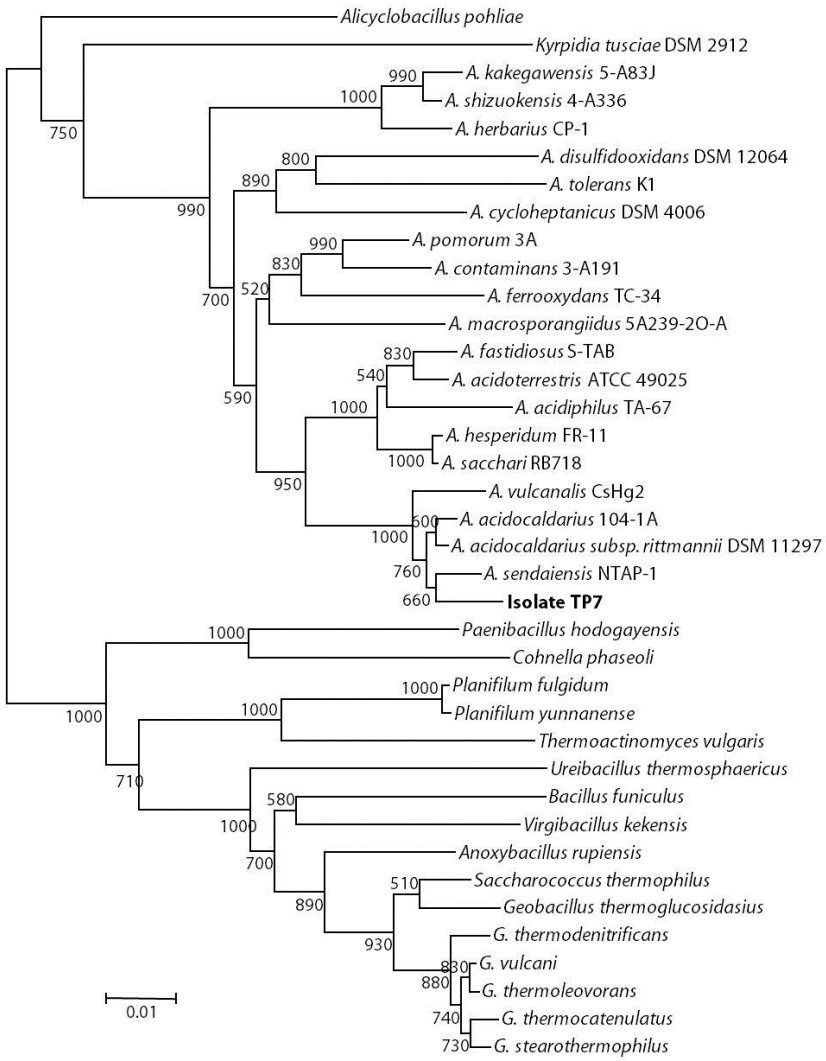

FIGURE S1. Phylogenetic analysis of isolate TP-7 based on its 16S rRNA gene sequence. The 16S rRNA gene (1489 bp) of isolate TP-7 was amplified by PCR. The amplified PCR products were sequenced and analyzed by using Clustal X ver. 1.81 (10). A phylogenetic tree was constructed using available 16S rRNA gene sequences of members of the *Alicyclobacillus*/ *Geobacillus* subphylum of thermophilic bacteria by the neighbor-joining method and p-distance methods (7). The phylogenetic position of isolate TP-7 was within the radiation of the genus *Alicyclobacillus*. The numbers indicate the bootstrap scores of 1000 trials. The bar equals 1 base substitution per 100 nt positions.

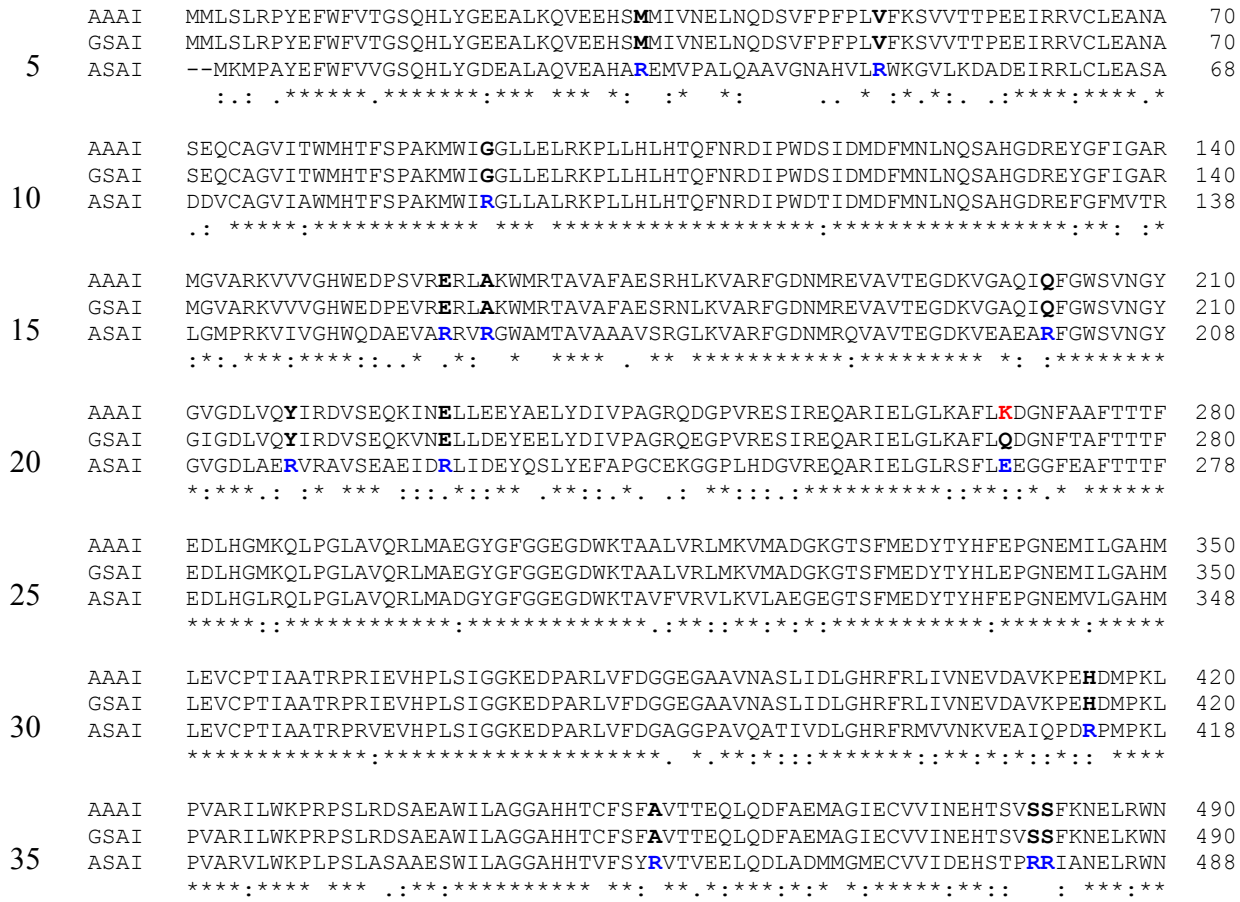

FIGURE S2. Alignment of the amino acid sequences of ASAI and its homologous AIs generated using CLUSTAL X (10). Non-conserved R residues are indicated in boldface blue type, and K268 of AAAI as the A residue in red font. ASAI, *A. acidocaldarius* TP7 AI (GenBank database accession no. JQ945232); GSAI, *Geobacillus stearothermophilus* AI (AAD45718); AAAI, *A. acidocaldarius* AI (AAY68209).

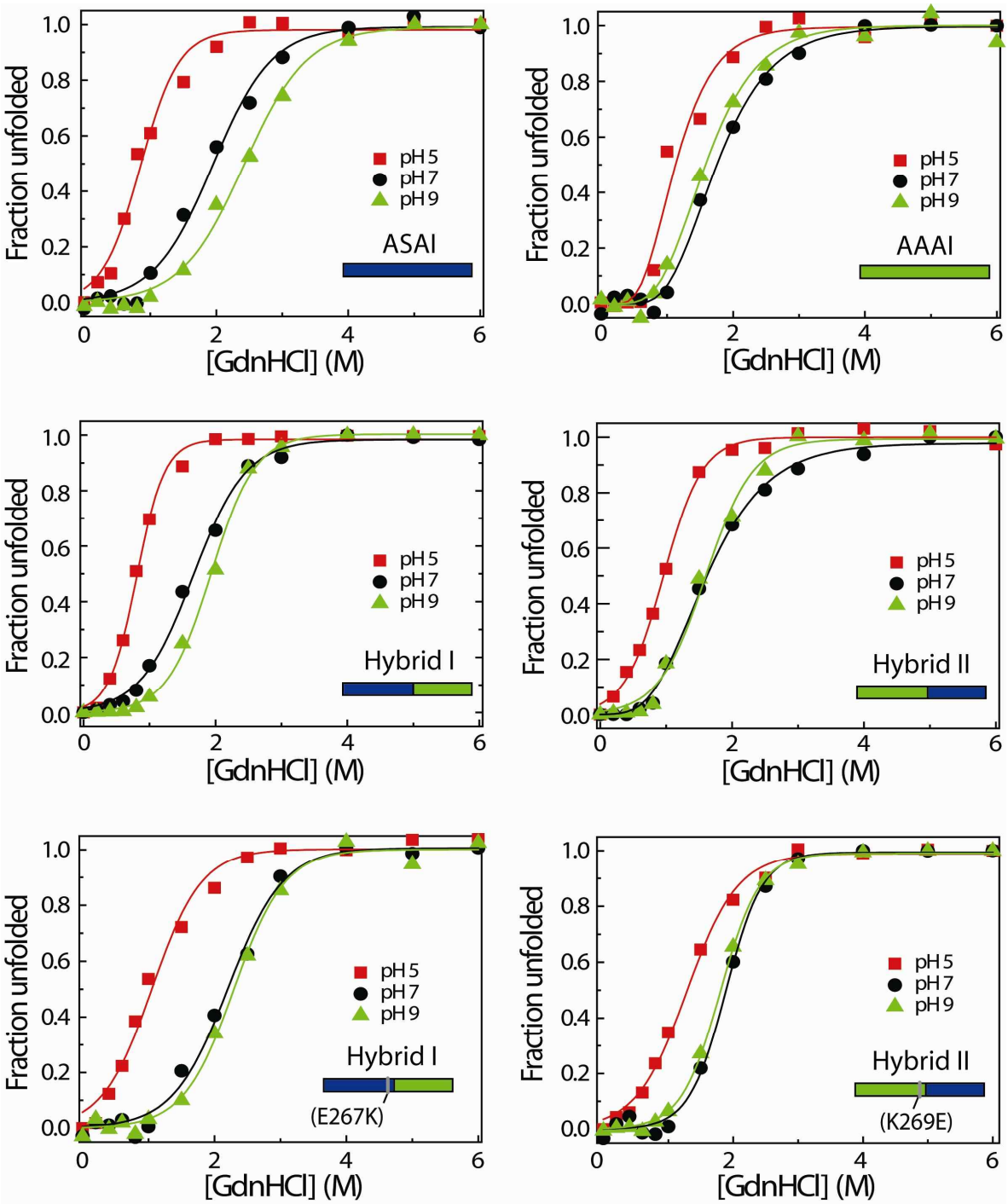

FIGURE S3. Equilibrium unfolding transitions of the wild-type AIs and their chimeras induced by GdnHCl at 25ºC monitored by CD ellipticity at 222 nm.

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