A naturally variable residue in the S1 subsite of M1-family aminopeptidases modulates catalytic properties and promotes functional specialization

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Running title: M1-aminopeptidase specificity

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Background: M1-family aminopeptidases exhibit a diverse range of specificities.

Results: Substitutions at a residue in the S1 binding pocket can induce structural changes and remodel specificity.

Conclusion: Mutations in the S1 subsite contribute to the acquisition of distinct specificities.

Significance: Variation of a key residue in the S1 binding pocket provides a pathway for the evolution of new specificities and functions.

SUMMARY

M1-family metallo-aminopeptidases fulfill a wide range of critical and in some cases medically relevant roles in humans and human pathogens. The specificity of M1-aminopeptidases is dominated by the interaction of the well-defined S1 subsite with the sidechain of the first (P1) residue of the substrate and can vary widely. Extensive natural variation occurs at one of the residues that contributes to formation of the cylindrical S1 subsite. We investigated whether this natural variation contributes to diversity in S1 subsite specificity. Effects of eleven substitutions of the S1 subsite residue valine 459 in the Plasmodium falciparum aminopeptidase PfA-M1 and of three substitutions of the homologous residue methionine 260 in Escherichia coli PepN were characterized. Many of these substitutions altered steady-state kinetic parameters for dipeptide hydrolysis and remodeled S1 subsite specificity. The most dramatic change in specificity resulted from substitution with proline, which collapsed S1 subsite specificity such that only substrates with P1-Arg, -Lys or -Met were appreciably hydrolyzed. The structure of PfA-M1 V459P revealed that the proline substitution induced a local conformational change in the polypeptide backbone that resulted in a narrowed S1 subsite. The restricted specificity and active site backbone conformation of PfA-M1 V459P mirrored those of endoplasmic reticulum aminopeptidase 2, a human enzyme with proline in the variable S1 subsite position. Our results provide compelling evidence that changes in the variable residue in the S1 subsite of M1-aminopeptidases have facilitated the evolution of new specificities and ultimately novel functions for this important class of enzymes.

Aminopeptidases of the M1 family are ubiquitous across the three kingdoms of life (1). These zinc-dependent enzymes catalyze the hydrolysis of the first amide bond of a peptide substrate. The M1 family has undergone an expansion in some higher eukaryotes (1) and is represented by 12 members in humans. Many of these have highly specialized roles and some are known to be medically important. For example, the mammalian ectoenzyme aminopeptidase N contributes to the metabolism of a number peptide
hormones and has been implicated in a wide variety of physiological processes including blood pressure regulation, angiogenesis and tumor metastasis (2). The endoplasmic reticulum aminopeptidases (ERAP) 1 and 2 trim peptides that are destined for major histocompatibility complex (MHC) class I presentation (3). These enzymes have been associated with ankylosing spondylitis, pre-eclampsia and cervical cancer (4). M1-aminopeptidases also play critical metabolic roles in human pathogens. The *Plasmodium falciparum* aminopeptidase PfA-M1 contributes to host hemoglobin catabolism in the parasite’s food vacuole (5-7) and has been validated as a drug target (8,9). In prokaryotes such as *Escherichia coli*, the M1-aminopeptidase PepN contributes to cytosolic peptide catabolism and to adaptation to nutritional downshift and high temperature stress (10).

Accompanying the functional diversity of M1-aminopeptidases is a high degree of variability in the specificity of the S1 subsite for the sidechain of the P1 residue of the substrate. The S1 subsite of M1-aminopeptidases is a well-defined pocket, in contrast to the more open S1’ and S2’ sites (11,12). Thus, the S1 subsite often plays a dominant role in defining the substrate range of the enzyme. An “archetypal” S1 specificity can be loosely defined as a preference for the basic residues Arg and Lys and non-β-branched non-polar residues over acidic, small polar and β-branched non-polar residues and proline. This specificity is observed for numerous well-characterized enzymes, including mammalian aminopeptidase N, prokaryotic PepN and PfA-M1 (13-16). There are, however, many examples of deviations from this archetypal specificity. ERAP2 exhibits a strong preference for the straight-chain residues Arg and Lys over all other natural amino acid sidechains (17,18). Conversely, the human enzyme “arginyl aminopeptidase-like 1” prefers hydrophobic and small polar P1 residues over Arg and Lys (19).

The diversity in S1 subsite specificities implies that this subsite is malleable and that its specificity can be modulated to accommodate a wide variety of functions. The architecture of the S1 subsite of *P. falciparum* PfA-M1 is depicted in Fig. 1B and C. Four residues (E319, V459, M462 and Y575; Fig. 1A) define a “cylinder” (13) that extends upward from the catalytic Zn(II) ion (these are referred to as “S1-cylinder residues” here). At the top of the cylinder are two “cap” (13) residues (E572 and M1034). When the identities of the S1-cylinder residues in all twelve human M1-human aminopeptidases, PfA-M1 and *E. coli* PepN were compared, it was apparent that one of the S1-cylinder residues (corresponding to V459 in PfA-M1) varies much more widely in size and polarity than do the other three residues (Fig. 1D, E). These observations raised the question of whether variation at this position of the S1 cylinder contributes to the diversity of S1 subsite specificities in M1-aminopeptidases.

Structural studies of *E. coli* PepN offer some support for this idea. The variable S1-cylinder residue in PepN, M260, changes conformation when the S1 subsite is occupied (13,20). In the unliganded state, M260 is found inside the S1 cavity. Upon binding of the inhibitor bestatin or of amino acids, the M260 sidechain swings out of the S1 subsite and can interact with the P1 sidechain. In addition, the polypeptide backbone around M260 moves outward by 0.8 Å, thereby expanding the S1 subsite (13). Movement of the M260 sidechain can also be driven by a bulky S2’ residue (11). Thus, both direct interactions between M260 and substrate sidechains as well as local conformational mobility of the backbone may contribute to defining the S1 specificity of PepN.

In this study, we tested the hypothesis that changes in the S1-cylinder residue corresponding to M260 in *E. coli* PepN and V459 in PfA-M1 can substantively modulate S1 subsite specificity. Eleven PfA-M1 variants with substitutions of V459 and three PepN variants with substitutions of M260 were generated. Specificities of the enzyme variants were characterized by determining the steady-state kinetics parameters for the hydrolysis of a panel of dipeptide substrates. The structural basis for the restricted specificity of the PfA-M1 variant with a proline substitution (V459P) was elucidated by solving the crystal structure of the enzyme-arginine complex. Comparisons with structures of other M1-aminopeptidases were undertaken to evaluate the importance of S1 subsite substitutions for the evolution of new specificities in natural M1-aminopeptidases.
EXPERIMENTAL PROCEDURES

Generation of PfA-M1 and PepN mutants

Mutations at codon 459 were introduced into a PfA-M1 expression plasmid (7) using a Quikchange mutagenesis kit (Stratagene). To generate an expression plasmid for *E. coli* PepN, the full-length open reading frame was PCR amplified from *E. coli* strain TOP10 (Invitrogen) genomic DNA and cloned into the BamH1 and NotI sites of pET45b, which introduced an N-terminal hexahistidine tag. The forward primer encoded a tobacco etch virus protease cleavage site immediately preceding the PepN sequence. Mutation of codon 260 to encode Val, Phe and Pro was accomplished by Quikchange mutagenesis. All sequences were confirmed by DNA sequencing.

Protein expression and purification and determination of Zn(II) stoichiometry

PfA-M1 and PepN variants were expressed in Rosetta2 *E. coli* (EMD Biosciences), which were grown in Luria-Bertani medium supplemented with 100 μM ZnCl2 to an optical density at 600 nm of ~0.9. Expression was induced with 1 mM isopropyl β-D-1-thiogalactoside for 4 hours at 25 °C. Cell pellets were lysed and the proteins were purified and treated with tobacco etch virus protease as previously described for wild-type PfA-M1 (14). Purified enzymes were snap-frozen in liquid nitrogen and stored at -80 °C. Protein concentrations were determined by absorbance at 280 nm using calculated extinction coefficients of 1.15 x 10^5 M^-1·cm^-1 (PfA-M1), 1.17 x 10^5 M^-1·cm^-1 (PfA-M1 V459Y), 1.21 x 10^5 M^-1·cm^-1 (PfA-M1 V459W) and 1.18 x 10^5 M^-1·cm^-1 (PepN). Determination of Zn(II) stoichiometries for purified PfA-M1 and PepN variants was conducted by ion chromatography as previously described (14).

Peptide cleavage assays

Dipeptide hydrolysis assays were conducted in 100 mM sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate pH 7.5/110 mM NaCl at 30 °C for 15 minutes. For each enzyme-substrate combination, assays to determine steady-state kinetic parameters were conducted using substrate concentrations ranging from ~0.2K_m to ~5K_m. Amino acid products were derivatized with AccQ•Tag (Waters) and analyzed by reverse phase ultra-high pressure liquid chromatography as previously described (14). Peak areas for the AccQ•Tag derivative of Ala (all X-Ala substrates) or Leu (Gly-Leu substrate) were determined using Empower2 software (Waters) and were converted to picomoles of amino acid by reference to an Ala or Leu standard. Plots of initial rate vs. substrate concentration were fit by non-linear regression to the Michaelis-Menten equation \( v = V_s/(K_m + s) \) using Kaleidagraph 4.1 (Synergy Software) where \( V \) is the limiting velocity and \( s \) is the substrate concentration. \( k_{cat} \) was calculated from the relationship \( V = k_{cat}[E] \). In most cases, data sets consisted of 9 substrate concentrations (including \( [S] = 0 \)) with a minority of data sets having 6-8 substrate concentrations. Steady-state kinetic parameters for wild-type PfA-M1 were reported previously (14).

In order to obtain kinetic parameters for a large number of enzyme/substrate combinations, in most cases these parameters are derived from single data sets. The quality of the Michaelis-Menten non-linear regression fits was monitored using the R^2 value, which describes how well the regression line fits the data (a value of 1 indicates that the regression line perfectly fits the data). For PfA-M1 variants, R^2 was equal to or greater than 0.98 for 86% of the Michaelis-Menten fits; the remainder ranged from 0.92 to 0.979. For *E. coli* PepN variants, all of fits were associated with an R^2 value greater than 0.97.

Structural analysis of PfA-M1 V459P-Arg

PfA-M1 V459P was crystallized following the published protocol used for wild-type PfA-M1 (21) with the addition of 1 mM L-arginine to the crystallization drop. Crystals were loop mounted without cryo-soaking and flash-frozen in liquid nitrogen. The dataset used for the structure solution was collected at beamline X29A (National Synchrotron Light Source, Brookhaven National Laboratory) using an ADSC Q315 CCD detector. A single crystal was used for data collection and structure solution. Data processing was carried out at the synchrotron beamline with the HKL2000 program suite (22). The structure was solved by molecular replacement using PHASER (23) and the structure of unliganded PfA-M1 (PDB 3EBG; (21)) as template. The model was manually adjusted against weighted

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difference Fourier maps using COOT and refined with REFMAC. Water molecules were added to the structure after several rounds of manual adjustment and refinement. Model quality was assessed with PROCHECK (24). All non-glycine residues resided either in the most favorable (97.9%) or in the allowed regions (2.1%) of the Ramachandran plot and the overall geometry was better than average when compared to structures solved at the same resolution. The atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession code 4J3B.

Comparisons of the V459P PfA-M1 structure to wild-type PfA-M1 or E. coli PepN (unliganded wild-type or with arginine or phenylalanine ligands) were made on the basis of global alignments. For comparison of PfA-M1 V459P and ERAP2, residues within a radius of 5 Å from the arginine ligand were used in the structural alignment.

RESULTS

Effects of substitutions at the S1 subsite residue V459 on the catalytic properties of PfA-M1

Valine 459 of PfA-M1 was replaced with 11 non-polar or uncharged polar amino acids: Gly, Ala, Ser, Thr, Leu, Ile, Met, Phe, Tyr, Trp and Pro. This set of mutations captured nearly all of the variation observed in the human enzymes at this position (Fig. 1D). Because PfA-M1 activity depends on a single active site Zn(II) ion (14,21), the Zn(II) stoichiometry for each enzyme variant was measured. The PfA-M1 variants bound between 1.1 and 1.5 equivalents of Zn(II) (Table 1), which indicates that amino acid substitutions at position 459 do not disrupt Zn(II) binding in the active site.

The effects of the substitutions on steady-state kinetic parameters were determined with four X-Ala dipeptide substrates that have been previously employed to define the “archetypal” S1 subsite specificity of wild-type PfA-M1 (14). Three dipeptides had hydrophobic P1 sidechains of varying size (Ala-Ala, Leu-Ala, Phe-Ala) and one had a basic P1-sidechain (Arg-Ala). Dipeptides are very likely to be physiological substrates of PfA-M1 in the food vacuole as they are generated from globin oligopeptides by the vacuolar exopeptidase dipeptidyl aminopeptidase 1 (25). There is also an experimental advantage in using dipeptides as the products of hydrolysis of dipeptides cannot (unlike those of longer peptides) serve as alternate substrates.

Substitutions at position 459 exerted a strong influence on both the Michaelis constants (K_m) and the turnover numbers (k_cat; Fig. 2) with the four X-Ala substrates. Two consistent trends in K_m values were evident: i) K_m values were among the lowest for all four substrates when residue 459 was aromatic, and ii) the K_m was high when position 459 was occupied by a proline residue.

Changes in k_cat values were generally much more modest than those of K_m (Fig. 2B) with the exception of Phe-Ala. A tradeoff between K_m and k_cat was observed for hydrolysis of Ala-Ala, Leu-Ala and Phe-Ala by PfA-M1 variants with a non-polar residue at position 459 (Fig. 2C). Such a tradeoff is expected from transition state theory if these substitutions affect the free energy of the ground state enzyme-substrate complex but not that of the transition state (26). In contrast, k_cat appeared to be largely independent of K_m for Arg-Ala (Fig. 2C), an outcome that suggests that the substitutions influence the free energies of both the ground and transition states. Thus, the effects of substitutions at the variable S1-cylinder residue on the energetics of the catalytic cycle can differ among P1 sidechains.

To illustrate how each substitution at position 459 affected PfA-M1 specificity, k_cat/K_m values were compared after normalizing to those of the wild-type enzyme (Fig. 3A). Perturbations in substrate specificity ranged from modest (V459A) to severe (V459W, V459P). The PfA-M1 variants could be divided by inspection into four groups, each of which contained enzymes with similar specificity profiles: i) G, A, S, T; ii) I, L, M; iii) F, Y, W; and iv) P (Fig. 3A). Notably, each group contained amino acids with sidechains of similar physico-chemical properties: i) small/polar; ii) aliphatic non-polar; iii) aromatic non-polar; iv) imino acid.

To acquire a more detailed picture of the specificity changes engendered by variation at position 459, representative members of these four groups (V459A, T, L, M, F and P) were each characterized with five additional substrates (Val-Ala, Met-Ala, Tyr-Ala, His-Ala and Gly-Leu; Fig. 3B). Gly-Leu, a dipeptide without a P1 sidechain, was chosen to determine the effects of the V459
substitutions on catalysis in the absence of interactions with the S1 subsite (a P1’ Leu residue was required for this substrate to lower the \( K_m \) to an experimentally tractable value (14)). Comparison of relative \( k_{cat}/K_m \) values for the expanded substrate set reveals that variation of the residue at position 459 can induce profound changes in PfA-M1 specificity (Fig. 3B). The V459P variant exhibited a remarkable shift in specificity such that substrates with unbranched P1 residues larger than Ala (Met, Arg) were much more efficiently cleaved than the others tested. Interestingly, the catalytic parameters for Gly-Leu hydrolysis were strongly perturbed by the V459P substitution (Fig. 3B, C), which indicates that this substitution alters the nature of enzyme-substrate interactions outside of the S1 subsite. More modest changes in residue 459 sidechain structure (V459L, M) also altered S1 specificity such that small (Gly, Ala) or large polar (His, Tyr) sidechains were more strongly preferred over aliphatic and basic ones. The V459F substitution increased catalytic efficiency across all substrates, whereas V459T generally suppressed catalytic efficiency. The V459A substitution effected only a modest change in specificity.

**Comparison of the effects of S1-cylinder mutations in PfA-M1 and PepN**

To assess the generality of the results obtained with PfA-M1, we characterized a small set of *E. coli* PepN mutants in which M260, the residue homologous to V459, was changed to Val, Phe or Pro. *E. coli* PepN was chosen for this experiment because its sequence has diverged substantially from that of PfA-M1 (50% identity over 250 aligned residues in the catalytic thermolysin-like domain II) yet it has retained the three other residues that define the S1-cylinder (E319, M462 and Y575 in PfA-M1 are structurally equivalent to E121, M263 and Y376 in PepN; Fig. 1A).

The Zn(II) stoichiometries of the four PepN variants (wild-type and three mutants) ranged from 1.8 to 2.6 Zn(II) atoms per enzyme (Table 1). These data are most consistent with a 2:1 Zn(II)-to-protein molar ratio. This result was initially surprising as structural analyses of PepN clearly revealed a single Zn(II) ion in the active site (13,20,27). However, in the high resolution PepN-Phe co-complex structure, two additional catalysis binding sites distal from the active site were reported (13). We speculate that our purification protocol, which involved incubating purified PepN with an excess of Zn(II), resulted in Zn(II) occupancy of one of these sites.

The specificity profile of each PepN variant was determined with a panel of six X-Ala substrates (Fig. 4A). Substitution at M260 resulted in substantial changes in enzyme specificity. Interestingly, the M260P substitution recapitulated the specificity for straight-chain P1 sidechains that was observed with PfA-M1 V459P. Catalytic efficiencies for PfA-M1 and PepN variants bearing identical configurations of S1-cylinder residues are compared in Fig. 4B. In general, PepN variants were better catalysts with P1-Arg and -Lys substrates than their PfA-M1 counterparts. Differences in S1 cap residues in the two enzymes, which are positioned at the top of the S1 subsite and in PepN interact with P1-Arg and -Lys sidechains (13), may be responsible for these specificity differences (see Discussion).

**Structural basis for the restricted specificity of PfA-M1 V459P**

We were intrigued by the marked shift in specificity to P1-Arg, -Lys and -Met that was effected by replacement of the variable S1-cylinder residue with proline in PfA-M1 and PepN. To determine the structural basis for this phenomenon, we solved the crystal structure of PfA-M1 V459P in complex with a molecule of arginine to 2.2 Å resolution (Table 2). Alignment of the structures of wild-type, unliganded PfA-M1 (21), PDB 3EBG) and PfA-M1 V459P-Arg yielded an 0.4 Å root-mean-square deviation for backbone atoms. In addition to the V459P mutation, we noticed that the residue H378 in the our structure is replaced with proline in the PfA-M1 structures reported by McGowan et al (21). This His-to-Pro change at residue 378 caused a repositioning of the backbone of residues 376-379 with a maximal displacement of 2.1 Å for the \( \alpha \)-carbon of residue 377 (data not shown). The genome sequence of the 3D7 clone of *P. falciparum* (28), which was the source of the PfA-M1 sequence used in this study, indicates that a histidine is encoded at residue 378 in this strain.

The active site of PfA-M1 V459P was occupied by a single arginine molecule (Fig. 5A, B). The amino and carboxylate groups of the Arg
laid form multiple interactions with the enzyme and the guanidinium group interacted with the carboxylate group of the S1-cap residue E572.

Comparison of the S1 subsite of unliganded wild-type PfA-M1 to that of the V459P-Arg structure revealed that the proline substitution caused the polypeptide backbone to move towards the center of the S1 subsite (Fig. 5C). This 1.1 Å movement of the α-carbon of residue 459 resulted in the Pro sidechain projecting into the S1 subsite, reducing its width. Notably, the positions of the other three S1 cylinder residues (E319, M462 and Y575) in the two structures were unchanged (Fig. 5C). The other noteworthy differences between the structures were the sidechain conformations of the two S1-cap residues, E572 and M1034. Upon binding of arginine, the sidechain of E572 moved toward the S1 subsite such that its carboxylate group was within hydrogen bonding distance of the Arg guanidinium group (Fig. 5B, C). In the wild-type PfA-M1 structure, the sidechain of M1034 adopts two conformations: one occupies the S1 subsite while the other swings away from it (21). In the PfA-M1 V459P-Arg structure, only the latter sidechain conformation was observed.

We asked whether the backbone movement observed in PfA-M1 V459P might be caused by Arg ligand binding by comparing its structure to that of the PepN-Arg co-complex determined by Addlagatta and colleagues (13). The positions of the Arg molecules in the S1 subsites were very similar in the two structures (Fig. 5D). Interestingly, the backbone positions around the homologous residues P459 (PfA-M1) and M260 (PepN) were very different, with a 1.0 Å distance between the α-carbons of the two residues (Fig. 5D).

How the V459P mutation might restrict entry of bulky P1 sidechains into the S1 pocket was evaluated by aligning the PfA-M1 V459P-Arg structure with Addlagatta’s structure of PepN complexed with Phe (13). If the P1-Phe sidechain were to adopt the same position in PfA-M1 V459P as it does in PepN, a steric conflict would arise between the phenyl ring and the P459 sidechain (Fig. 5E). Clearly this steric conflict can be resolved, as the $K_m$ of PfA-M1 V459P for Phe-Ala is only 2.3-fold higher than that for the wild-type enzyme; however, the 85-fold decrease in the $k_{cat}$ value suggests that the position of Phe-Ala in the active site of PfA-M1 V459P is sub-optimal for catalysis of amide bond hydrolysis.

To determine whether the structural changes induced by proline substitution in PfA-M1 V459P might represent a general mechanism for constricting S1 subsite specificity, we compared its S1 subsite structure to that of the recently reported crystal structure of ERAP2 (29). ERAP2 is a human aminopeptidase with a naturally-occurring Pro residue (P333) in the homologous position. Although these two enzymes share only 26% identity in the catalytic domain, the position of the polypeptide backbone around P333 of ERAP2 was remarkably similar to that around V459P (Fig. 5F). Notably, all four S1-cylinder residues adopted highly similar positions in the two enzymes (Fig. 5F). Like PfA-M1 V459P, ERAP2 exhibits a high specificity for substrates with P1-Arg and -Lys residues (17,18). Our structural comparison suggests that a conformational change in the polypeptide backbone induced by P333 contributes to defining the specificity of ERAP2.

**DISCUSSION**

In this study, we examined the effects of varying an S1 subsite cylinder residue on the catalytic properties of two M1-family aminopeptidases. All of the substitutions at V459 of PfA-M1 and M260 of PepN, including potentially destabilizing mutations to Gly, Pro and residues with large sidechains such as Trp, were tolerated and did not perturb Zn(II) binding. Several features likely account for the ability to accommodate a wide range of substitutions at this S1 subsite residue. The polypeptide backbone at this position does not adopt a regular secondary structure (20,21,27) and is able to undergo local conformational changes, as shown in the structure of PfA-M1 V459P presented here and reported previously for PepN (13). Conformational flexibility in the position of the sidechain, as has been observed for M260 in PepN (20,27), may also mitigate any potential steric clashes arising from the substitutions.

Many of the eleven substitutions of V459 in PfA-M1 altered the catalytic properties of the enzyme as determined by the hydrolysis of a panel of dipeptide substrates with varying P1-sidechain structures. Values for both $K_m$ and $k_{cat}$ were influenced by the nature of the substituted S1-
cylinder sidechain, with both parameters exhibiting changes of up to two orders of magnitude for a single substrate across the twelve PfA-M1 variants. These findings suggest ways in which substitutions at the variable S1-cylinder residue could provide an adaptive benefit to M1-aminopeptidases evolving new functions. Substitutions could serve to align the $K_m$ of the enzyme with the *in vivo* substrate concentration. For example, an aminopeptidase having a peptide hormone as a substrate, a low $K_m$ value (such as we observe when a large non-polar residue is placed at position 459 in PfA-M1) might be more important than a high $k_{cat}$ value. Alternately, turnover number ($k_{cat}$) may be the critical parameter to optimize in situations where substrate concentrations are high and rapid substrate turnover is required, such as in the PfA-M1 catalyzed release of amino acids from hemoglobin-derived peptides in the *P. falciparum* food vacuole (7). In such cases, $k_{cat}$ could potentially be maximized if a residue with a small sidechain (Ala, Ser, Thr, Val) were present in the variable S1-cylinder position. Such an argument implies that replacement of V459 with Met or Phe would impair the *in vivo* functions of PfA-M1. We are investigating this by introducing mutations at position 459 into the chromosomal PfA-M1 sequence in *P. falciparum*.

Extensive specificity profiling of six PfA-M1 variants and three PepN variants revealed that changes in the identity of the variable S1-cylinder residue can modify S1 subsite specificity. The most dramatic change in specificity occurred upon substitution of proline: both PfA-M1 V459P and PepN M260P were highly selective for substrates with P1-Arg, -Lys and -Met. More subtle changes in specificity were also observed; for example, substitution of Leu at position 459 of PfA-M1 resulted in an order of magnitude shift in specificity in favor of substrates with P1-Gly, -His and -Tyr in comparison to those with P1-Arg and -Met. In some cases, the changes in specificity ($k_{cat}/K_m$) were less dramatic than the changes in $K_m$ and $k_{cat}$ due to the tradeoff between these parameters (e.g., PfA-M1 V459F).

Characterization of pairs of PfA-M1 and PepN variants with identical sets of the four S1-cylinder residues revealed the presence of a second means of tuning S1 subsite specificity. We observed that the PepN variant usually exhibited substantially higher catalytic efficiencies with P1-Arg and -Lys substrates and lower catalytic efficiencies with the large hydrophobic P1-residues Phe and Met than its paired PfA-M1 variant. Interactions with the S1-cap residues likely lie at the root of this difference. The cap residues are E572 and M1034 in PfA-M1 and N373 and Q821 in PepN. In the PepN-Arg crystal structure, both cap residues stabilize the binding of the Arg sidechain through hydrogen bonding to the guanidinium group (13). Replacement of Q821 with M1034 in PfA-M1 eliminates one of these hydrogen bonds. The role of M1034 may be to stabilize the binding of substrates with large, hydrophobic P1 residues through van der Waals interactions, which have been observed between M1034 and the hydrophobic P1-sidechains of the peptidic inhibitors bestatin and Co4 (Fig. 1B, C; (21)). We are generating S1-cap variants of PfA-M1 and PepN to decipher the roles of cap residues in stabilizing (or destabilizing) the binding of polar and hydrophobic P1-sidechains.

Structural analysis of PfA-M1 V459P with Arg in the active site revealed that the proline substitution induced a local change in conformation of the polypeptide backbone that created a narrower S1 subsite. This structural change appeared to affect the interaction of the enzyme with a dipeptide substrate in complex ways, as the kinetic parameters of PfA-M1 V459P with the substrate Gly-Leu, which lacks a P1 sidechain, were highly perturbed. Is it possible that Arg binding, and not the Pro substitution, is responsible for the backbone movement observed in the PfA-M1 V459P structure? Several lines of evidence suggest that this is unlikely. First, binding of the inhibitors bestatin and Co4 to wild-type PfA-M1 did not alter the position of the polypeptide backbone in the region of V459 (21). Second, binding of Arg to wild-type PepN induced a 0.8 Å outward (rather than inward) shift of the backbone around the homologous residue, M260, which enlarged the S1 pocket (21). Third, the close similarities in the structures of the S1 subsites of PfA-M1 V459P and ERAP2 suggest that the presence of a proline residue in the S1 cylinder determines the particular local backbone conformation observed in these distantly-related enzymes. We conclude that there are at least two ways in which substitutions of the variable S1-cylinder residue could impact S1 subsite
specificity: i) by altering the repertoire of direct interactions with substrate P1-sidechains, and ii) by influencing the local conformation of the polypeptide backbone. Whether substitutions other than proline affect the backbone conformation remains to be determined.

The striking similarities in the restrictive S1 subsite preferences of PfA-M1 V459P, PepN M260P and ERAP2 for P1-Arg and -Lys sidechains suggest that the presence of P333 in the S1-cylinder of ERAP2 has been a critical factor in shaping its biological function. The primary role of both ERAP1 and 2 is to trim proteasome-generated peptides in the endoplasmic reticulum for loading onto class 1 MHC molecules (3,4). The specificity of the S1 subsite of ERAP1 is relatively broad, accepting a wide range of acidic, basic and non-polar P1 sidechains (30,31). Its activity is regulated by a “molecular ruler” mechanism that strongly favors peptides longer than eight amino acids (32). This mechanism is important for generating peptides of an appropriate size for MHC loading. The S1 specificity of ERAP2 appears to complement that of ERAP1. ERAP2 is required to remove basic residues from the N-termini of some peptides destined for MHC presentation (33). However, ERAP2 activity is not length-restricted (32). Thus, the exclusion of many or most peptides (i.e., those not having N-terminal Arg or Lys residues) from the active site of ERAP2 may be important to prevent over-digestion of peptides to lengths too short for MHC loading. Our results support the idea that P333 constrains the S1 subsite of ERAP2, thereby restricting the enzyme’s repertoire of substrates in the endoplasmic reticulum.

Taken together, our results support the hypothesis that natural variation at a residue in the S1 subsite of M1-aminopeptidases can modulate enzyme specificity. Certainly other S1 subsite residues, such as the cap residues, can make an important contribution to overall specificity as well. Nevertheless, the case of ERAP2 provides compelling evidence that natural variation of the S1-cylinder residue studied here has had a role to play in promoting the functional specialization of M1-aminopeptidases.

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**FOOTNOTES**

1 Abbreviations: APA, APB, APN, APO, APQ, aminopeptidases A, B, N, O and Q, respectively; APBL, aminopeptidase B-like; ERAP, endoplasmic reticulum aminopeptidase; IRAP, insulin regulated aminopeptidase; LTA4H, leukotriene A4 hydrolase; MHC, major histocompatibility complex; PepN, aminopeptidase N; PfA-M1, *Plasmodium falciparum* M1-aminopeptidase; PSA, puromycin-sensitive aminopeptidase; TRHDE, thyrotropin-releasing hormone-degrading ectoenzyme.

2 The nomenclature of Schechter and Berger (34) is used here. P1, P1’ and P2’ refer to substrate residues while S1, S1’ and S2’ refer to the corresponding enzyme subsites. For aminopeptidases, the scissile peptide bond is between the P1 and P1’ residues.
FIGURE LEGENDS

Figure 1. One S1-cylinder residue is highly variable in M1-aminopeptidases. A, Schematic diagram of the two M1-family aminopeptidases characterized in this study, P. falciparum PfA-M1 and E. coli PepN. The four domains are indicated with roman numerals. The residues that comprise the cylinder of the S1 subsite are indicated above (PfA-M1) and below (PepN) in red. The 194-residue N-terminal extension of PfA-M1, which is not conserved in M1-aminopeptidases and was not present in the recombinant protein, is not shown. Scale, 1 cm = 200 amino acids. B, Stereo view of the S1 subsite from the structure of PfA-M1 complexed with the peptidic inhibitor bestatin (green) (PDB 3EBH; (21)). The four cylinder residues are colored light blue, the two cap residues are magenta and the Zn(II) atom is a dark blue sphere. The P1-phenyl ring of bestatin occupies the S1 subsite. C, Surface topology of the S1 subsite of PfA-M1 complexed with bestatin (cyan) illustrates its cylindrical shape. The surface surrounding bestatin was derived from those PfA-M1 residues within 5 Å of the ligand. The phenyl ring of bestatin occupies the S1 subsite. Residues that form the S1 subsite are labeled. D, The S1-cylinder residue corresponding to V459 in PfA-M1 is highly variable in homologous aminopeptidase sequences. An alignment of the XGAMEN motif of twelve human M1-family aminopeptidases and of P. falciparum PfA-M1 and E. coli PepN is shown, where X is the naturally variable S1-cylinder residue (shaded pink). Abbreviations: APA, APB, APN, APO, APQ, aminopeptidases A, B, N, O and Q, respectively; APBL, aminopeptidase B-like; ERAP1, ERAP2, endoplasmic reticulum aminopeptidase 1 and 2, respectively; IRAP, insulin regulated aminopeptidase; LTA4H, leukotriene A4 hydrolase; PSA, puromycin-sensitive aminopeptidase; TRHDE, thyrotropin-releasing hormone-degrading ectoenzyme. E, Weblog diagram (35) illustrating the sequence diversity at the four S1-cylinder residues across the M1-aminopeptidases shown in D. Sizes of letters are proportional to relative abundance. From left to right, the residues correspond to E319, V459, M462, Y575 in PfA-M1. Parts B and C were prepared using the PyMOL Molecular Graphics System.

Figure 2. Effects of substitution at residue 459 in PfA-M1 on the steady-state kinetic parameters $K_m$ and $k_{cat}$. A, $K_m$ values are represented with blue bars and the scale at left and $k_{cat}$ values with red bars and the scale at right. The substrate is indicated in the upper right of each panel. The identity of the residue at position 459 is indicated on the abscissa (wild-type = V). B, Fold-change of $K_m$ and $k_{cat}$ values for each of the four dipeptide substrates expressed as the ratio of the maximal value to the minimal value. C, Plots of $k_{cat}$ vs. $K_m$ for Phe-Ala, Leu-Ala, Ala-Ala and Arg-Ala for PfA-M1 variants with non-polar residues at position 459 (G, A, V, I, L, M, F, Y, W). The square of the Pearson correlation coefficient ($R^2$) is reported for each linear regression fit to indicate the extent of the correlation between $k_{cat}$ and $K_m$.

Figure 3. Effects of substitution at residue 459 in PfA-M1 on specificity. A, Relative $k_{cat}/K_m$ values (normalized to those for wild-type PfA-M1) for eleven PfA-M1 variants and four substrates. B, Relative $k_{cat}/K_m$ values for representatives of the four groups of PfA-M1 variants and nine substrates. The inset displays the data for the V459P variant with an expanded ordinate scale (0 – 0.8). For both A and B, the identity of the residue at position 459 is indicated on the abscissa and bars below the abscissa indicate the grouping of variants with similar specificity profiles. The horizontal line indicates a value of 1, i.e. identity with the wild-type $k_{cat}/K_m$ value. C, Values of $K_m$ (blue) and $k_{cat}$ (red) for hydrolysis of Gly-Leu by selected PfA-M1 variants

Figure 4. Effects of substitution at residue 260 in PepN on specificity and comparison of PfA-M1 and PepN variants with identical S1-cylinder residues. A, Relative $k_{cat}/K_m$ values (normalized to those for wild-type PepN) for three PepN variants and six substrates. The horizontal line indicates a value of 1, i.e. identity with the wild-type $k_{cat}/K_m$ value. B, Comparison of $k_{cat}/K_m$ values for pairs of PfA-M1 and PepN variants having identical configurations of S1-cylinder residues. Enzyme identities are indicated in the upper left corner of each panel.
Figure 5. Structural analysis of PfA-M1 V459P complexed with arginine. A, Omit map of the arginine molecule in the PfA-M1 V459P-Arg co-crystal structure at a contour level of 2σ. The Zn(II) atom (black sphere) is shown for orientation. B, LigPlot diagram (36) of the interactions between PfA-M1 V459P and the active site-bound arginine molecule. Hydrogen bonds are indicated with dashed green lines and distances in Å and hydrophobic interactions are indicated with red crescents. C, Stereo diagram comparing the S1 subsite in wild-type (blue) and V459P (magenta) PfA-M1. All S1 cylinder and cap residues are shown except for Y575, which was omitted for clarity. A second conformation of M1034 in wild-type PfA-M1, which is very similar to the position of that residue in PfA-M1 V459P, has also been omitted. Residue labels are for PfA-M1 V459P. D, Comparison of the S1 subsites of PfA-M1 V459P-Arg (magenta) and PepN-Arg (green; PDB 3B2P (13)). S1 cylinder residues are shown except Y575/Y376, which was omitted for clarity. Residue labels are shown for PfA-M1 V459P (upper) and PepN (lower). The Arg ligand (“Arg”) is indicated. E, Alignment of the structures of PfA-M1 V459P (magenta; Arg has been omitted for clarity) and E. coli PepN with phenylalanine (labeled “Phe”) in the active site (green; PDB 3B34; (13)). The sidechains of the four residues comprising the S1 cylinder are shown. The dashed black line indicates the closest distance (1.9 Å) between carbon atoms of the phenyl ring of the Phe molecule and the sidechain of P459. F, Comparison of the S1 subsites of wild-type PfA-M1 (cyan), PfA-M1 V459P (magenta) and ERAP2 (yellow; PDB 3SE6). S1-cylinder sidechains are shown for the latter two structures. The Arg ligand from the PfA-M1 V459P co-crystal structure is shown. The Lys ligand from the ERAP2 structure (29) was omitted for clarity. In C-F, Zn(II) atoms are represented with spheres. Parts A and C-F were prepared using the PyMOL Molecular Graphics System.
Table 1: Zn(II) stoichiometries for PfA-M1 and PepN variants.
Values are reported as the mean ± standard deviation from triplicate analyses.

| Protein | Variant | Zn(II) stoichiometry |
|---------|---------|----------------------|
| PfA-M1  | wild-type | 1.2 ± 0.1 |
|         | V459A   | 1.3 ± 0.1 |
|         | V459S   | 1.4 ± 0.1 |
|         | V459T   | 1.2 ± 0.1 |
|         | V459I   | 1.4 ± 0.1 |
|         | V459L   | 1.1 ± 0.1 |
|         | V459M   | 1.3 ± 0.1 |
|         | V459F   | 1.3 ± 0.1 |
|         | V459Y   | 1.4 ± 0.1 |
|         | V459W   | 1.6 ± 0.1 |
|         | V459P   | 1.5 ± 0.1 |
|         | V459G   | 1.4 ± 0.1 |
| PepN    | wild-type | 1.8 ± 0.1 |
|         | M260V   | 2.0 ± 0.2 |
|         | M260F   | 1.9 ± 0.2 |
|         | M260P   | 2.6 ± 0.2 |
Table 2: Statistics for the structure of PfA-M1 V459P complexed with L-arginine.

| Data Collection |  |
|-----------------|---|
| Molecules/asymmetric unit | 1 |
| Wavelength (Å) | 1.0750 |
| Space group | P2₁2₁2₁ |
| Unit cell parameters (Å) | a =76.0; b = 109.5; c = 112.9 |
| Resolution (Å) | 50-2.2 |
| Total reflections | 2,358,549 |
| Unique reflections | 63,876 |
| Completeness (%) | 99.6 (97.6) |
| Average I/σ | 18 (3.3) |
| Rmerge (%) | 11 (66) |

| Refinement |  |
|------------|---|
| Resolution range (Å) | 50-2.2 |
| R (%) | 16.2 |
| Rfree (%) | 20.1 |
| Root mean square deviation bond lengths (Å) | 0.005 |
| Root mean square deviation angles (º) | 0.84 |
| Average temperature factor (Å²) | 18.3 |
| Number of protein atoms | 7,300 |
| Number of solvent molecules | 438 |

* The values in parentheses relate to the highest resolution shell from 2.28-2.2Å.
Figure 1
Amino acids at position 459 of PfA-M1

| Substrate       | Max/min ratio | $K_m$ (mM) | $k_{cat}$ (s^-1) |
|-----------------|---------------|------------|------------------|
| Ala-Ala         | 140           | 3.5        |
| Leu-Ala         | 83            | 10         |
| Phe-Ala         | 60            | 97         |
| Arg-Ala         | 18            | 3.9        |

Figure 2
Figure 5
A naturally variable residue in the S1 subsite of M1-family aminopeptidases modulates catalytic properties and promotes functional specialization
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