Mutations at the S1 Sites of Methionine Aminopeptidases from *Escherichia coli* and *Homo sapiens* Reveal the Residues Critical for Substrate Specificity*  

Jing-Ya Li‡, Yong-Mei Cui‡, Ling-Ling Chen‡, Min Gu‡, Jia Li‡, Fa-Jun Nan‡‡, and Qi-Zhuang Ye‡‡‡  

From the 3Chinese National Center for Drug Screening, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, China and 4The High Throughput Screening Laboratory, Higuchi Biosciences Center, University of Kansas, Lawrence, Kansas 66047  

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Methionine aminopeptidase (MetAP) catalyzes the removal of methionine from newly synthesized polypeptides. MetAP carries out this cleavage with high precision, and Met is the only natural amino acid residue at the N terminus that is accepted, although type I and type II MetAPs use two different sets of residues to form the hydrophobic S1 site. Characteristics of the S1 binding pocket in type I MetAP were investigated by systematic mutation of each of the seven S1 residues in *Escherichia coli* MetAP type I (EcMetAP1) and human MetAP type I (HsMetAP1). We found that Tyr-65 and Trp-221 in EcMetAP1, as well as the corresponding residues Phe-197 and Trp-352 in HsMetAP1, were essential for the hydrolysis of a thiopeptolide substrate, Met-S-Gly-Phe. Mutation of Phe-191 to Ala in HsMetAP1 caused inactivity in contrast to the full activity of EcMetAP1(Y62A), which may suggest a subtle difference between the two type I enzymes. The more striking finding is that mutation of Cys-70 in EcMetAP1 or Cys-202 in HsMetAP1 opens up the S1 pocket. The thiopeptolides Leu-S-Gly-Phe and Phe-S-Gly-Phe, with previously unacceptable Leu or Phe as the N-terminal residue, became efficient substrates of EcMetAP1(C70A) and HsMetAP1(C202A). The relaxed specificity shown in these S1 site mutants for the N-terminal residues was confirmed by hydrolysis of peptide substrates and inhibition by reaction products. The structural features at the enzyme active site will be useful information for designing specific MetAP inhibitors for therapeutic applications.

Removal of the N-terminal methionine from newly synthesized polypeptides is an important posttranslational modification and is catalyzed by methionine aminopeptidase (MetAP).  

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‡ To whom correspondence may be addressed: Chinese National Center for Drug Screening, 189 Guo-Shou-Jing Road, Shanghai 201203, China. Tel.: 86-21-5080-1313; Fax: 86-21-5080-0721; E-mail: fj nan@mail.shcnc.ac.cn.

‡‡ To whom correspondence may be addressed: High Throughput Screening Laboratory, University of Kansas, 1501 Wakarusa Dr., Lawrence, KS 66047. Tel.: 785-330-4390; Fax: 785-330-4532; E-mail: qye@ku.edu.

‡‡‡ The abbreviations used are: MetAP, methionine aminopeptidase; Met, a product from Met removal, and Met analogs (methionine phosphonate, norleucine phosphonate, methionine phosphinate, norleucine phosphonate, methionine phosphonate; GST, glutathione S-transferase; MetP, 1-amino-3-(methylmercapto)propylphosphonic acid; MOPS, 4-morpholinepropanesulfonic acid; HPLC, high pressure liquid chromatography.
nate, and trifluoromethionine) all bind in a similar fashion at the active site of EcMetAP1, which defines the S1 site (8). The peptidic inhibitor AHHpA-Ala-Leu-Val-Phe-OMe, with the Nle residue for substrates. Available structural information indicates that they share 43% identity (114 of 264 residues) in both offer the same stringent specificity for the N-terminal residue for substrates. The S1 site residues that are important for substrate hydrolysis and substrate specificity are shown in boldface type.

Fig. 1. Sequence alignment of type I MetAPs from Escherichia coli (EcMetAP1), Saccharomyces cerevisiae (ScMetAP1, catalytic domain only), and Homo sapiens (HsMetAP1, catalytic domain only). The S1 residues mutated in this study are indicated by a ♦, and the conserved metal ligands are indicated by guest on July 24, 2018http://www.jbc.org/Downloaded from

expression for E. coli (EcMetAP1), S. cerevisiae (ScMetAP1, catalytic domain only), and Homo sapiens (HsMetAP1, catalytic domain only). All of the Xaa-S-Gly-Phe thiopeptolide substrates, where Xaa represents Met, Leu, Phe, or Nle. The S1 residues mutated in this study are indicated by a ♦, and the conserved metal ligands are shown in boldface type.
The $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values were derived from non-linear regression fitting of the curve in the plot of initial rates versus substrate concentrations (up to 10 mM) by using the Michaelis-Menten equation. The $IC_{50}$ values were obtained by nonlinear curve fitting of the plot of initial rates versus inhibitor concentrations according to the following equation: percentage of inhibition $= 100/(1 + (IC_{50}/[I])^k)$, where $[I]$ is the inhibitor concentration and $k$ is the Hill coefficient.

**HPLC Assays Using Tripeptides Xaa-Ala-Ser**—Hydrolysis of Xaa-Ala-Ser tripeptide substrates was assayed by an HPLC method, and substrate depletion was determined by absorbance at 215 nm after the substrate and the products were separated by HPLC. Peak area was used to calculate the hydrolysis of the substrate. All assays were performed at room temperature in a 20-$\mu$l reaction system containing 50 mM MOPS, pH 7, 50 $\mu$M CoCl$_2$, 8 mM tripeptide, and 5 $\mu$M EcMetAP1 (wild-type or a mutant) for 5 min or 2 $\mu$M HsMetAP1 (wild-type or a mutant) for 30 min. The reaction was quenched by the addition of 80 $\mu$l of 0.1% trifluoroacetic acid, and the mixture was centrifuged at 10,000 $\times$ g for 10 min. A reverse-phase C8 column (Eclipse, XDB; 5 $\mu$m, 4.6 $\times$ 150 mm) was used for HPLC analysis. Mobile phase A consisted of water containing 0.1% trifluoroacetic acid, and mobile phase B consisted of 1:1 water to acetonitrile plus 0.1% trifluoroacetic acid. A 10-$\mu$l sample was loaded onto the column by 5% phase B at a flow rate of 1 ml/min.

**RESULTS**

**Preparation of Wild-type and Mutant MetAP1 Enzymes**—The S1 site residues in EcMetAP1 (Cys-59, Tyr-62, Tyr-65, Cys-70, His-79, Phe-177, and Trp-221) have been identified from available x-ray structures (Fig. 2) (7, 8, 12). However, there is no structural information available for HsMetAP1. Both EcMetAP1 and HsMetAP1 belong to the type I MetAP family and share 43% sequence identity (Fig. 1). Six of the seven S1 site residues identified in EcMetAP1 are either identical or conserved in HsMetAP1, and, therefore, it is likely that HsMetAP1 has a structure similar to that of EcMetAP1. We prepared nine single point mutants of EcMetAP1 (C59A, C59S, Y62A, Y65A, C70A, C70S, H79A, F177A, and W221A) by replacing each of the seven residues with Ala or Ser, and seven single point mutants of HsMetAP1 (P191A, Y194A, F197A, C202A, H211A, and W352A) by replacing each of the seven residues with Ala or Ser, and seven single point mutants of HsMetAP1 (P191A, Y194A, F197A, C202A, H211A, and W352A) by replacing each of the seven residues with Ala or Ser, and seven single point mutants of HsMetAP1 (P191A, Y194A, F197A, C202A, H211A, and W352A) by replacing each of the seven residues with Ala or Ser, and seven single point mutants of HsMetAP1 (P191A, Y194A, F197A, C202A, H211A, and W352A) by replacing each of the seven residues with Ala or Ser, and seven single point mutants of HsMetAP1 (P191A, Y194A, F197A, C202A, H211A, and W352A) by replacing each of the seven residues with Ala or Ser, and seven single point mutants of HsMetAP1 (P191A, Y194A, F197A, C202A, H211A, and W352A) by replacing each of the seven residues with Ala or Ser, and seven single point mutants of HsMetAP1 (P191A, Y194A, F197A, C202A, H211A, and W352A) by replacing each of the seven residues with Ala or Ser, and seven single point mutants of HsMetAP1 (P191A, Y194A, F197A, C202A, H211A, and W352A) by replacing each of the seven residues with Ala or Ser, and seven single point mutants of HsMetAP1 (P191A, Y194A, F197A, C202A, H211A, and W352A) by replacing each of the seven residues with Ala or Ser. The Km, $k_{cat}$, and $k_{cat}/K_m$ values for these enzymes were determined and are shown in the figure. The data show that the mutants have similar catalytic efficiencies to the wild-type enzymes, suggesting that the S1 site residues are not critical for catalysis. The Km values for the mutants are comparable to the wild-type values, indicating that the mutations do not significantly alter substrate binding. The $k_{cat}$ values for the mutants are also similar to the wild-type values, suggesting that the mutations do not significantly alter catalytic efficiency. The $k_{cat}/K_m$ values for the mutants are comparable to the wild-type values, indicating that the mutations do not significantly alter the catalytic efficiency per substrate molecule.
F308A, and W352A) were prepared by replacing each of the seven corresponding residues (Pro-191, Tyr-194, Phe-197, Cys-202, His-211, Phe-308, and Trp-352) with Ala.

Wild-type and mutant EcMetAP1s were expressed in E. coli as soluble proteins and purified to homogeneity with high yield. Recombinant HsMetAP1s, both wild-type and mutant, were expressed in E. coli as GST-fusion proteins and purified with GSTrap affinity column. HsMetAP1 proteins were released from the fusion proteins by thrombin treatment. Based on SDS-PAGE analyses, the purity of wild-type and mutant Ec-MetAP1 or Hs-MetAP1 was >95%.

**Residues Important for Hydrolysis of Met-S-Gly-Phe**—Seven EcMetAP1 mutants with each S1 residue replaced by Ala were tested on the known substrate Met-S-Gly-Phe first. Three residues (Tyr-65, His-79, and Trp-221), when replaced by Ala, had huge impact on EcMetAP1 activity in hydrolyzing Met-S-Gly-Phe. EcMetAP1(Y65A) and EcMetAP1(W221A) became totally inactive, whereas EcMetAP1(H79A) had very weak activity (Fig. 3). Tyr-65 and Trp-221 are located near each other and have direct contact with the terminal methyl group of the Met side chain (Fig. 2). Their inactivity may indicate that hydrophobic interaction with the methyl group is required for binding and hydrolysis of a substrate. His-79 is in the same vicinity and close to the catalytic metal site, although it is not a metal ligand. In the case of the bestatin-based inhibitor (7), His-79 interacts with atoms in the peptide bond between the P1' and P2' residues. However, in the transition state analogs (8) this His moves toward the metal center with the potential to form hydrogen bonds with the atoms of the scissile peptide bond (P1 and P1' residues). Therefore, the causes for significantly reduced activity of EcMetAP1(H79A) may be more complex.

On the other hand, the other four residues (Cys-59, Tyr-62, C70A, and F177A), when replaced by Ala, showed minimal effect on the activity. EcMetAP1(C59A) and EcMetAP1(Y62A) were as active as the wild-type, and EcMetAP1(C70A) and EcMetAP1(F177A) showed only slightly reduced activity. However, a closer look revealed that both EcMetAP1(C59A) and EcMetAP1(C70A) showed increased $K_m$, along with increased $k_{cat}$, so that the overall effect on $k_{cat}/K_m$ was minimal. All four residues are located on one side of the pocket (Fig. 2).

When corresponding mutants of HsMetAP1 were tested, we found that some results were consistent with those from EcMetAP1 mutants (Fig. 3). Mutation of Phe-197 (Tyr-65 in EcMetAP1) rendered the enzyme inactive, and mutation of Trp-352 (Trp-221 in EcMetAP1) displayed a significant reduction in activity. HsMetAP1(Y194A) was even more active than the wild-type enzyme, indicating that Pro-191 (Cys-59 in EcMetAP1) is not essential. Cys-59 is unique to EcMetAP1 and is not conserved in HsMetAP1 and other MetAPs.

HsMetAP1(C202A), like EcMetAP1(C70A), also showed both increased $K_m$ and increased $k_{cat}$ as compared with wild-type HsMetAP1, indicating its active participation in substrate hydrolysis.

However, differences also emerged. Significant disagreement exists between HsMetAP1(Y194A) and EcMetAP1(Y62A). HsMetAP1(Y194A) displayed no activity in hydrolyzing Met-S-Gly-Phe in contrast to the full activity shown by EcMetAP1(Y62A). This disagreement reflects the subtle differences.

**Fig. 4. Hydrolysis of Met-S-Gly-Phe (A and D), Leu-S-Gly-Phe (B and E), or Phe-S-Gly-Phe (C and F) by wild-type and S1 site mutants of EcMetAP1 and HsMetAP1.**
at the S1 site of MetAPs from two different species, which is an important feature that we are exploring for specific MetAP inhibitors.

Residues Important for Maintaining Stringent Specificity for Terminal Met as Revealed by Using Thiopeptolides Xaa-S-Gly-Phe—It is intriguing how the stringent specificity for a terminal Met is conferred by the S1 site residues. The side chain of Met is largely hydrophobic, with a sulfur atom as a potential hydrogen bond acceptor. However, the sulfur is not involved in hydrogen bonding in the x-ray structures (8). Because Nle-Gly-Gly (4) and Nle-pNA (5) are validated substrates, the side chain of Nle can fit into the pocket as well as that of Met. It is likely that hydrophobic interaction and steric exclusion strongly influence the ability of the S1 pocket to discriminate between Met and other residues. To better probe the pocket, we prepared 14 thiopeptolides with the general structure of Xaa-S-Gly-Phe (Xaa = Ala, Asn, Asp, Gly, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Tyr, Trp, or Val), based on the known substrate Met-S-Gly-Phe.

None of the 14 thiopeptolides (Xaa is a natural amino acid residue but not Met) could be cleaved by wild-type EcMetAP1 or HsMetAP1, except for Thr-S-Gly-Phe, which showed barely detectable hydrolysis (7.3 μmol/min/μM) by HsMetAP1. This result confirms the stringent substrate specificity for the N-terminal residue.

When these thiopeptolides were tested on the S1 site mutants, significant hydrolysis of some thiopeptolides was detected (Fig. 4). Leu-S-Gly-Phe was cleaved readily by EcMetAP1(C70A) and weakly by EcMetAP1(C59A) and EcMetAP1(Y62A). Cys-59 and Tyr-62 in EcMetAP1 are the same two residues that showed no adverse effect on hydrolysis of Met-S-Gly-Phe upon mutation to Ala (Figs. 3 and 4). The effect of the mutation of Cys-70 to Ala on the hydrolysis of Leu-S-Gly-Phe is interesting, because it greatly increased both \( K_m \) and \( k_{\text{cat}} \) in the hydrolysis of Met-S-Gly-Phe. EcMetAP1(C70A) hydrolyzed Leu-S-Gly-Phe (\( K_m = 0.35 \pm 0.04 \) mM; \( k_{\text{cat}} = 20.8 \pm 1.0 \) s\(^{-1}\); \( k_{\text{cat}}/K_m = 59,088 \) s\(^{-1}\) M\(^{-1}\)) as efficiently as wild-type EcMetAP1 hydrolyzed Met-S-Gly-Phe (\( K_m = 0.35 \pm 0.01 \) mM; \( k_{\text{cat}} = 22.5 \pm 0.3 \) s\(^{-1}\); \( k_{\text{cat}}/K_m = 63,757 \) s\(^{-1}\) M\(^{-1}\)). The importance of this Cys in controlling specificity is even more noticeable in HsMetAP1 mutants, and HsMetAP1(C202A) (corresponding to EcMetAP1(C70A)) was the only one among the seven S1 site mutants that cleaved Leu-S-Gly-Phe.

The structural difference between the side chains of Met and Leu is a branch at the γ-carbon in Leu. Conversion of this Cys (Cys-70 in EcMetAP1 and Cys-202 in HsMetAP1) to Ala opens up a space to accommodate the methyl group at the γ-carbon. From the results of this study, it is not known how the side chain of Leu fits actually into the pocket after the Cys to Ala mutation. The x-ray structures of EcMetAP1 complexed with a series of Met analogs (methionine phosphonate, norleucine phosphonate, methionine phosphonate, trifluoromethionine, and Met) (8), indicating that there is no significant rotation of the bonds of the side chain. Indeed, based on the structure of EcMetAP1 complexed with 1-amino-3-(methylmercapto)propylphosphonic acid (MetP), we can fit the methyl group toward this Cys without rotating any bonds of the side chain (Fig. 5). Phe is another amino acid residue with a branch at the γ-carbon, and Phe-S-Gly-Phe was cleavable to a significant extent only by EcMetAP1(C70A) or HsMetAP1(C202A) among the S1 site mutants (Fig. 4).

Replacement of Cys by Ala could also disrupt hydrogen bonds due to the removal of the thiol group. To reduce disruption to the S1 site, we also made two Cys to Ser mutants, namely EcMetAP1(C59S) and EcMetAP1(C70S). The side chain of Ser is considerably smaller than that of Cys (OH versus SH), and the hydroxyl group in Ser could function as a hydrogen bond donor or acceptor similar to the thiol group in Cys. The fact that EcMetAP1(C70S) cleaved both Leu-S-Gly-Phe (\( K_m = 0.14 \pm 0.01 \) mM; \( k_{\text{cat}} = 4.04 \pm 0.26 \) s\(^{-1}\); \( k_{\text{cat}}/K_m = 29,154 \) s\(^{-1}\) M\(^{-1}\)) and Phe-S-Gly-Phe (\( K_m = 0.24 \pm 0.01 \) mM; \( k_{\text{cat}} = 4.55 \pm 0.22 \) s\(^{-1}\); \( k_{\text{cat}}/K_m = 17,500 \) s\(^{-1}\) M\(^{-1}\)) at efficiencies similar to that for Met-S-Gly-Phe (\( K_m = 0.68 \pm 0.02 \) mM; \( k_{\text{cat}} = 30.8 \pm 0.6 \) s\(^{-1}\); \( k_{\text{cat}}/K_m = 45,541 \) s\(^{-1}\) M\(^{-1}\)) suggests that the space created by changing Cys to Ser, although not optimal, is already enough for the side chain of Leu or Phe of a substrate to fit into the S1 site for efficient cleavage.

Confirmation of Change of Substrate Specificity of S1 Site Mutants by Using Tripeptides Xaa-Ala-Ser—Because of the heightened reactivity intrinsic to the thiopeptolides, it becomes crucial to parallel the more convenient thiopeptolide assay with a true peptide-based assay to minimize kinetic bias. We prepared four tripeptides, namely Met-Ala-Ser, Leu-Ala-Ser, Phe-Ala-Ser, and Nle-Ala-Ser, and tested them on the four S1 site mutants that showed activity on the thiopeptolides Leu-S-Gly-Phe and Phe-S-Gly-Phe, along with wild-type enzymes (Table I).

Met-Ala-Ser has been reported as an efficient substrate (\( K_m = 0.38 \) mM; \( k_{\text{cat}} = 210 \) min\(^{-1}\); \( k_{\text{cat}}/K_m = 550,000 \) s\(^{-1}\) M\(^{-1}\)) for HsMetAP2 (5). Under our assay conditions, it was hydrolyzed by wild-type EcMetAP1 and the EcMetAP1(C59A), EcMetAP1(Y62A), and EcMetAP1(C70A) mutants, and wild-type HsMetAP1 and the HsMetAP1(C202A) mutant. This is consistent with the results from the thiopeptolides, which show that these mutations do not affect the cleavage of a substrate with Met as the P1 residue. It was not surprising to see that Ile-Ala-Ser was hydrolyzed by the wild-type and mutant enzymes listed in Table I, because other data already suggested that the Nle side chain is able to occupy the S1 pocket comfortably (4, 5).

It caused some concerns when we did not observe significant hydrolysis of Leu-Ala-Ser by the mutants tested. The difference between Leu-Ala-Ser and Leu-S-Gly-Phe, which is an excellent substrate, is that an amide bond in the peptide is replaced by a thioester bond in the thiopeptolide, in addition to the variations
S1 Site Mutants of Methionine Aminopeptidases

Hydrolysis of Xaa-Ala-Ser by EcMetAP1, HsMetAP1, and their mutants

| Enzyme        | Met-Ala-Ser | Leu-Ala-Ser | Phe-Ala-Ser | Nle-Ala-Ser |
|---------------|-------------|-------------|-------------|-------------|
| EcMetAP1      | 100         | <4          | <4          | 113         |
| EcMetAP1(C59A)| 97          | <4          | <4          | 115         |
| EcMetAP1(Y62A)| 94          | <4          | <4          | 116         |
| EcMetAP1(C70A)| 31          | <4          | 39          | 56          |
| HsMetAP1      | 100         | <9          | <9          | 113         |
| HsMetAP1(C202A)| 76         | <9          | 61          | 99          |

* Rates of hydrolysis of Met-Ala-Ser by EcMetAP1 and HsMetAP1 were 248 ± 9 and 116 ± 2 μm/min/μM, respectively. The detection limit was 10 μm/min/μM under our assay conditions.

Inhibition of hydrolysis of Met-S-Gly-Phe by EcMetAP1, HsMetAP1, and their mutants

| Enzyme        | IC₅₀[^a] Met | IC₅₀[^a] Leu | IC₅₀[^a] Phe | IC₅₀[^a] Ile |
|---------------|-------------|-------------|-------------|-------------|
| EcMetAP1      | 28.2 ± 2.6  | (0)         | (8 ± 3)     | (0)         |
| EcMetAP1(C59A)| 33.0 ± 3.9  | (19 ± 3)    | (0)         | (0)         |
| EcMetAP1(C59S)| 32.3 ± 4.3  | (25 ± 2)    | (0)         | (0)         |
| EcMetAP1(Y62A)| 35.6 ± 2.7  | (33 ± 8)    | (9 ± 3)     | (0)         |
| EcMetAP1(C70A)| 30.4 ± 3.5  | (45 ± 5)    | 4.1 ± 0.2   | (10 ± 2)    |
| EcMetAP1(C70S)| 28.1 ± 1.5  | (40 ± 3)    | 10.2 ± 0.9  | (6 ± 1)     |
| HsMetAP1      | 7.1 ± 0.8   | (0)         | (7 ± 4)     | (7 ± 1)     |
| HsMetAP1(C202A)| 61.1 ± 5.4  | (45 ± 3)    | (58 ± 2)    | (0)         |

[^a]: Percentage of inhibition at 74 mM Leu, 110 mM Phe, or 110 mM Ile is in parentheses.

at the P1’ and P2’ residues. This HPLC assay is certainly not a sensitive assay, but, nevertheless, it is still an adequate working tool for confirming the results obtained from the thiopeptolides. It is significant that Phe-Ala-Ser was cleaved by EcMetAP1, tested (Table II). Met also binds to MetAP, showing detectable activity. This result clearly demonstrates that a mutation at Cys-70 in HsMetAP1(C202A). Met to reaction product dissociates from the enzyme. The affinity of MetAP removes Met from peptide substrates, and Met as a chain of Phe not only for a thiopeptolide substrate but also for a peptide substrate.

Product Inhibition of Wild-type and Mutated MetAP1s— MetAP removes Met from peptide substrates, and Met as a reaction product dissociates from the enzyme. The affinity of Met to EcMetAP1 and HsMetAP1 was evaluated for its competition with Met-S-Gly-Phe by the colorimetric assay. Met binds only weakly to EcMetAP1 as indicated by its IC₅₀ at 28.2 mM, and it binds equally weakly to the EcMetAP1 mutants tested (Table II). Met also binds to HsMetAP1 and the mutant HsMetAP1(C202A).

If the role of Cys-70 of EcMetAP1 or Cys-202 of HsMetAP1 is in maintaining the stringent specificity is critical, Leu or Phe as a product from peptide cleavage could conceivably fit into the S1 site also. Phe binds the most strongly to EcMetAP1(C70A) and relatively strongly to EcMetAP1(C70S) and has almost no binding to wild-type EcMetAP1. This order of binding affinity correlates very well with the gradual enlargement of the S1 pocket caused by changing the residue at position 70 from Cys to Ser to Ala. Although the hydrolysis of Leu-Ala-Ser cannot be detected and the affinity of Leu to the enzymes is weak, the rank order of the binding of Leu to wild-type and mutant EcMetAP1s and HsMetAP1s is in good agreement with the data from thiopeptolides and peptides. Ile cannot fit into the S1 pocket, and its inability to bind to the enzymes was expected and confirmed.

DISCUSSION

By removing the N-terminal Met from nascent polypeptides, MetAP plays a crucial role in protein maturation for proper localization, targeting and eventual degradation in many normal and pathological processes. MetAP has been recognized as a promising target for antibacterial, antifungal and anticancer agents (18).

In eukaryotic cells, type I and type II MetAPs are redundant, and only the deletion of both genes in yeast Saccharomyces cerevisiae is lethal (19, 20). Antiangiogenic fumagillin and its analogues specifically target HsMetAP2 (the type II human enzyme) without affecting the type I isozyme HsMetAP1 (21, 22), and a semisynthetic derivative, TNP-470, is in clinical trials for cancer therapy (23). Many pathogenic bacteria have only one MetAP, which is type I, and its presence is essential for cell survival. Its essential role has been demonstrated by the lethal deletion of the single MetAP gene in E. coli (24) and S. typhimurium (25). For minimal toxicity, it is desirable to design an inhibitor specific for bacterial MetAP without affecting the human type I enzyme HsMetAP1. Understanding the role of each residue at the enzyme active site in the recognition of substrates and inhibitors and the cleavage of the amide bond in the substrate is crucial in designing a MetAP inhibitor with the desired specificity for therapeutic applications.

Both type I and type II MetAPs have the same “pita bread” fold, and all of the five metal ligands are conserved (12). Marine natural products called bengamides inhibit tumor growth in vivo and in vitro, and their molecular target was recently identified as MetAP (26). LAF389, a synthetic analog, inhibited both type I and type II human MetAPs at submicromolar concentrations. However, selective MetAP inhibitors can be obtained as demonstrated by fumagillin, which covalently modifies His-231 of HsMetAP2 by opening an epoxide ring (11). Covalent modification of the conserved His in the type I enzyme EcMetAP1 (His-79) was achieved only at a much higher concentration (27).
It is more challenging to discover inhibitors that discriminate among type I MetAPs, the important targets for antibacterial drug development. The residues forming the S1 site are conserved among type I MetAPs such as EcMetAP1 and HsMetAP1 (Fig. 1), and they shape a pocket that accommodates the side chain of Met or Nle. Side chains of other amino acids with a significant difference in size may be barred simply by steric exclusion, and their binding will not produce a productive hydrolytic event. None of the 14 thiopeptolide Xaa-S-Gly-Phe (Xaa is a natural amino acid residue other than Met) substrates was cleavable to a significant extent by either EcMetAP1 or HsMetAP1, confirming the stringent specificity for Met as the terminal residue.

Both Met and Nle have a straight side chain. There are several natural amino acids with a side chain of similar size, and from our data it is likely that Cys-70 in EcMetAP1 or Cys-202 in HsMetAP1 functions as a “gate keeper” to prevent a side chain with a branch, such as that of Leu, Ile, Val, or Phe, from fitting into this pocket. Mutation of this Cys to Ala makes it possible for side chains with a branch at the γ-carbon to fit into the pocket, and Leu-S-Gly-Phe and Phe-S-Gly-Phe become cleavable substrates. The side chain of Val and Ile with a branch at the β-carbon was still not able to fit into the S1 site.

Mutants C70S, W221L, and C70S/W221A of EcMetAP1 have been reported previously (28), and they had 54, 27, and 6% activity, respectively, in hydrolyzing Met-Gly-Met-Met as compared with wild-type EcMetAP1. In contrast to the inactivity of EcMetAP1(Y65A) that we observed in this study, the Y65F mutant kept the same hydrolytic activity on the peptide (28). The change from Tyr to Phe was not as dramatic as the change from Trp to Ala, which may account for this discrepancy. Our data confirm the importance of Trp-221 in the hydrolysis of a substrate with Met as the P1 residue and additionally reveal the crucial role of Cys-70 in maintaining stringent specificity at the S1 site. Our systematic mutation of all S1 site residues, combined with thiopeptolide and peptide substrates as S1 site probes, has revealed valuable information about the contribution of each residue to substrate recognition and hydrolysis.

The S1’ site also plays an important role in substrate recognition, which is shallow and only accepts small and uncharged amino acid residues at the penultimate position of substrates. Walker and Bradshaw reported a mutagenesis study on the S1’ site of ScMetAP1 (29), and Met-329 (Met-206 in EcMetAP1), Gln-356 (Gln-233 in EcMetAP1), and Ser-195 (Ser-72 in EcMetAP1) were all mutated to Ala. It is remarkable that the mutants Q356A and M329A were able to cleave N-terminal Met from substrates that have large penultimate residues, an observation similar to what we report here.

There are few inhibitors for bacterial and fungal MetAPs reported in the literature. The substrate-like inhibitor AHHPa-Ala-Leu-Val-Phe-O-Me inhibited EcMetAP1 with an IC₅₀ of 5 μM (30). We recently discovered, by using high throughput screening of a diverse compound library, a series of nonpeptidic small molecules that inhibit EcMetAP1 specifically and show no inhibition for HsMetAP1 (13, 31). We are in the process of defining their binding mode on the enzyme and are investigating how these inhibitors exploit the structural features on the enzyme for such specificity. The information gained from this and other studies of the MetAP enzyme active site will shed light on the eventual development of MetAP inhibitors as useful therapeutic agents.

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Jing-Ya Li, Yong-Mei Cui, Ling-Ling Chen, Min Gu, Jia Li, Fa-Jun Nan and Qi-Zhuang Ye

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