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Amino Acid Residues Involved in the Functional Integrity of *Escherichia coli* Methionine Aminopeptidase

CHEN-HSIANG CHIU,[1] CHAO-ZONG LEE,[1] KUNG-SHIH LIN,[1] MING F. TAM,[2] AND LIH-YUAN LIN[1]*

Institute of Radiation Biology and Department of Life Science, National Tsing Hua University, Hsinchu,[1] and Institute of Molecular Biology, Academia Sinica, Nankang, Taipei,[2] Taiwan, Republic of China

Received 10 March 1999/Accepted 24 May 1999

Amino acid residues in the metal-binding and putative substrate-binding sites of *Escherichia coli* methionine aminopeptidase (MAP) were mutated, and their effects on the function of the enzyme were investigated. Substitution of any amino acid residue at the metal-binding site resulted in complete loss of the two cobalt ions bound to the protein and diminished the enzyme activity. However, only Cys70 and Trp221 at the putative substrate-binding site are involved in the catalytic activity of MAP. Changing either of them caused partial loss of enzyme activity, while mutations at both positions abolished MAP function. Both residues are found to be conserved in type I but not type II MAPs.

Methionine aminopeptidase (MAP) (EC 3.4.11.18) is a metallic enzyme that removes the initiator methionine from nascent proteins (3). However, not all proteins are subjected to this cleavage. The initiator methionine can be completely removed from the nascent protein only when the penultimate amino acid has radii of gyration of 1.29 Å or less, i.e., glycine, alanine, proline, serine, cysteine, threonine, and valine (11, 15). The proteins may be subjected to further modification after removal of the initiator methionine (1).

MAP acquires cobalt ions to achieve maximum enzymatic activity in vitro (3). Whether cobalt ions are sequestered in vivo by the enzyme remains unclear. It has been shown recently that MAP can also use Zn^{2+} as a cofactor (17). MAP has been found in *Escherichia coli* (3), *Pyrococcus furiosus* (16), *Methanococcus jannaschi* (4), *Salmonella typhimurium* (12), *Bacillus subtilis* (13), *Haemophilus influenzae* (6), *Saccharomyces cerevisiae* (5, 9), swine (7), and humans (2). Based on primary structural characteristics, MAPs are classified into two types (2). Interestingly, human and porcine MAPs (type II) have been demonstrated to be the previously reported initiator factor-2-associated protein (p^{67}) (2, 10), and they have the same substrate specificities as do type I MAPs. However, whether type II MAPs isolated from other species (e.g., microorganisms) play the same role as that of the human or porcine enzyme remains uncertain. Both types of MAPs are present in *S. cerevisiae* (5, 9), while only one type of MAP has been identified in other species. Whether two types of MAPs exist in all organisms requires further investigation.

The X-ray structure of the cobalt-dependent MAP from *E. coli* has been elucidated (14). It displays a structure that differs from that of other proteolytic enzymes. The metal ions are liganded by the side chains of five amino acid residues in an approximately octahedral coordination. by sequence alignment, these five residues are conserved among all MAPs known so far, including p^{67} (16). This result implies that these residues play essential functional roles and have been conserved through evolution.

A hydrophobic pocket that is considered to be important for substrate binding is located adjacent to the metal-binding site (14). The importance of the metal-binding site and that of the substrate-binding site have not been characterized. A recent study showed that a mutation of the *S. cerevisiae MAP1* gene corresponding to one of the amino acid residues in the metal-binding site of *E. coli* MAP resulted in 10^3-fold-lower catalytic activity (K_{cat}/K_m). In addition, overexpression of the mutated protein in *S. cerevisiae* caused a slow-growth phenotype and interfered with the wild-type MAP1 in a dominant manner (8). In this work, we mutated all of the metal-binding residues and six amino acids located in the putative substrate-binding sites of the *E. coli* MAP. The enzymatic activity of these mutants was investigated.

The MAP gene was amplified by PCR with *E. coli* genomic DNA used as a template. Primers used in this reaction were synthesized according to the first and last 20 nucleotides of the reported *E. coli* MAP gene sequence (3). The product was extracted by phenol-chloroform, precipitated by ethanol, and resuspended in water. The amplified product contains not only the MAP coding sequence but also the promoter region. The product was then digested by *Ava*I to remove part of the 5′ region and ligated with a pBS(+) vector (Stratagene). Before ligation, the vector was digested with *Eco*RI. After filling of the cohesive ends with Klenow enzyme, the resulting product was digested again with *Ava*I. The MAP gene was then inserted into the vector with the desired orientation. Since this construct contains the MAP promoter, the MAP gene can be transcribed constitutively after being transformed into *E. coli* host cells (JM109).

Mutated MAP genes were obtained by PCR. Products obtained by amplifying the template with 100 pmol of mutagenic primer and primer I (Table 1) were separated electrophoretically in a 0.8% agarose gel. A DNA fragment of the expected size was eluted off the gel with a Jetsorb kit (Genomed) and served as a megaprimer for the second PCR. In the second PCR, the primers in the previous reaction were replaced by 20 pmol of megaprimer and 100 pmol of primer II (Table 1). The wild-type and mutated MAP genes generated by PCR were cloned and sequenced to ensure that there were no unexpected nucleotide changes in the coding region. The plasmids were then transformed into *E. coli*, and the genes were expressed constitutively. MAP can be produced in large quantities (approximately 5% of the cytosolic protein) in this *E. coli* expression system.

The procedures for MAP purification followed those de-

* Corresponding author. Mailing address: Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan, ROC. Phone: 886-3-5742693. Fax: 886-3-5715934. E-mail: lylin@rb.nthu.edu.tw.

TABLE 1. Primer sequences used in site-directed mutagenesis

Mutant	Mutagenic primer[a]	Orientation[b]	Primer I[c]	Primer II[d]
Metal-binding site				
D97A	TACGGTGACAgCAATGGTAAC	A	R	U
D108A	TTTCGAGGTAgCGCCGTGGAA	A	R	U
H171L	TATTGCGGACtCGGTATTGGT	S	U	R
E204V	TTCACCATCGtGCCAATGGTC	S	U	R
E235V	GCACAATATGtGCATACTAAT	S	U	R
Hydrophobic pocket				
C59S	GTTTCTGCCaGCCTCGGCTAT	S	R	U
C70S	AATAGAGATGCtAACGGATTT	A	U	R
Y62F	ATAGCCGTGAaAGCCGAGGCA	A	U	R
Y65F	GGATTTCGGAaAGCCGTGATA	A	U	R
F177L	GGTCGCGGCTTgCATGAAGAA	S	R	U
W221L	AAAGATGGCTtGACGGTAAAA	S	R	U

[a] Nucleotides that create the desired mutation are presented in lowercase letters.

[b] Orientation of the mutagenic primer corresponding to the sense (S) or antisense (A) strand of the *E. coli* MAP gene.

[c] Primers that are paired with the mutagenic primer for PCR for generation of the megaprimer.

[d] Primers that are paired with the megaprimer for the second PCR. U, universal primer (5′-GTAAAACGACGGCCAGC-3′); R, reverse primer (5′-GGAAACAGCTATGACCATG-3′).

scribed by Ben-Bassat et al. (3), with modifications. The transformed cells were cultured overnight in 500 ml of Luria-Bertani broth supplemented with 50 μg of ampicillin per ml. After centrifugation at 1,500 × *g* for 15 min, the cell pellet was resuspended in 30 ml of 0.2 M potassium phosphate (pH 7.5)–0.2 mM $CoCl_2$–0.1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed by sonication, and debris was removed by centrifugation at 15,000 × *g* for 20 min. The supernatant was applied to a DEAE-Sepharose column equilibrated with buffer A (20 mM potassium phosphate [pH 7.5], 0.2 mM $CoCl_2$, 0.1 mM PMSF). A linear gradient of 0 to 0.25 M NaCl (in buffer A) was employed to elute the proteins. Fractions containing MAP were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme activity assay. The MAP fractions (eluted at 0.1 M NaCl) were pooled and concentrated to approximately 300 μl by ultrafiltration (Amicon) and applied to a Superose 12 gel filtration column (HR10/30; Pharmacia) equilibrated and eluted with buffer B (0.1 M potassium phosphate [pH 7.5], 0.2 mM $CoCl_2$, 0.1 mM PMSF). The MAP fractions from the gel filtration column were pooled again and diluted with 4 volumes of deionized water. The solution was subsequently loaded onto a Mono-Q column (HR5/5; Pharmacia) preequilibrated in buffer A (20 mM potassium phosphate [pH 7.0], 0.1 mM dithiothreitol, 1 mM methionine). A linear gradient of 0 to 0.25 M NaCl (in buffer C) was used to elute the MAP. In this step, cobalt was not added to the buffer. The concentration of purified protein was determined with a protein assay kit (Bio-Rad). The cobalt content of the purified protein was determined by inductively coupled plasma mass spectrometry, with buffer C as a control. Activity of the enzyme was assayed according to the procedures described by Ben-Bassat et al. (3). For determination of enzyme kinetic parameters, the initial velocity from the linear portion of the reaction progress curve was analyzed for different concentrations of substrate (from 90 to 630 μM, with increments of 45 μM). Values of K_m and V_{max} were determined from double-reciprocal plots of initial reaction velocity as a function of substrate concentration.

Compared to the procedures described by Ben-Bassat et al. (3), we utilized an additional Mono-Q column to enhance the purity of the enzyme in our preparation. The MAP was purified to homogeneity, as demonstrated by SDS-PAGE (data not shown). Since deletion of the MAP gene is lethal to *E. coli* (3), we cannot employ MAP-null cells to produce mutant MAP. Fortunately, the quantity of MAP is very limited in host cells, since no MAP activity can be detected from 500 ml of cell culture by the purification procedures described above. In contrast, at least 1 mg of MAP can be obtained from the same volume of cells transformed with a plasmid carrying the MAP gene (either wild type or mutant).

The amino acid residues involved in cobalt binding were mutated individually. The enzymatic activity of each mutant was examined after purification. As shown in Table 2, almost complete loss of enzymatic activity was observed for proteins carrying a substitution at any amino acid residue associated with metal binding. Compared to the wild-type enzyme, only 4, 2, 6, 5, and 3% residual activity was observed for D97A (i.e., a change from D to A at position 97), D108A, H171L, E204V, and E235V mutants, respectively.

Besides the enzyme activity, the cobalt-binding capacity of the mutants was determined. We employed an anion-exchange (Mono-Q) column in the final step of our purification scheme. Cobalt was omitted from the elution buffer, and the purified MAPs should not contain free cobalt in the solution. After determination of protein concentration, the cobalt content of the purified MAPs was analyzed by inductively coupled plasma mass spectrometry. The Co^{2+}/protein ratio of wild-type MAP was calculated to be 1.6. The results are in agreement with those obtained by X-ray crystallographic analysis, which indicates the presence of two cobalt ions in each MAP molecule (14). However, the cobalt content was almost undetectable for the mutant MAPs. The cobalt content was reduced to 0, 6, 3, 0, and 3% of that of the wild type for D97A, D108A, H171L, E204V, and E235V mutants, respectively (Table 2). The results indicate that the integrity of the metal-binding site is essential for maintaining the catalytic activity and interactions of both cobalt ions with MAP.

In addition to residues in the metal-binding site, selected constituents of the hydrophobic pocket were also mutated. The

TABLE 2. Relative enzyme activities and cobalt contents for wild-type and mutant *E. coli* MAPs

Enzyme	Relative activity (%)	Relative cobalt content (%)[a]
Metal-binding site		
Wild type	100	100
D97A	4	ND
D108A	2	6
H171L	6	3
E204V	5	ND
E235V	3	3
Hydrophobic pocket		
C59S	108	
Y62F	110	
Y65F	98	
C70S	54	
F177L	123	
W221L	27	
C70 W221	6	

[a] The relative cobalt content was determined by comparing the amount of metal in the mutant with that of the same amount of wild-type MAP. ND, not detectable.

TABLE 3. Kinetic parameters for wild-type and mutated *E. coli* MAPs[a]

Enzyme	K_m (μM)	V_{max} (nmol · s^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Wild type	214.4	23.2	1.16	5.42×10^3
C70S	457.1	15.5	0.78	1.69×10^3
W221L	655.9	13.5	0.67	1.03×10^3

[a] Kinetic parameters were determined by using a peptide with the sequence Met-Gly-Met-Met as the substrate. Ten micrograms of protein was used in this assay.

hydrophobic pocket is considered to be associated with substrate binding and catalytic activity. The six amino acid residues that line this pocket, as proposed by Roderick and Matthews (14), were mutated individually, and the enzymatic activity of the mutants was analyzed. As revealed in Table 2, four of the mutant MAPs did not show a decrease in catalytic activity compared to that of the wild-type enzyme. The relative activity was 108, 110, 98, and 123% for C59S, Y62F, Y65F and F177L mutants, respectively. However, the relative activity was reduced to 54 and 27% of the wild-type level for C70S and W221L, respectively. Combining the mutations at C70 and W221 resulted in a protein with less than 6% of the wild-type MAP activity. These data demonstrate that C70 and W221 are probably required for the integral function of substrate binding and hence for the catalytic activity of MAP.

To further demonstrate the contribution of these residues in enzyme activity, we mutated both C59 and C70 and observed a drop of activity to 49% of that of the wild-type enzyme, which is similar to that of a C70 mutation alone. We also substituted a residue next to the hydrophobic pocket (H63L) and generated a double mutation at H63 and C59. Both mutant proteins showed catalytic activities similar to that of wild-type MAP (data not shown). These findings imply again that only C70 and W221 in the hydrophobic pocket are related to substrate binding and enzyme activity.

The enzyme kinetics of the wild-type, C70S, and W221 MAPs were analyzed and compared. As shown in Table 3, changes in both K_m and V_{max} occurred after mutation. Among them, W221L has the highest K_m and lowest V_{max}. The K_m of the enzyme increased approximately two- and threefold upon C70 and W221 mutation, respectively, while V_{max} decreased by only 35 to 42%. The results further support the notion that C70 and W221 are involved in substrate binding. Due to the low catalytic activity, kinetic parameters were not determined for mutants carrying a substitution at the metal-binding site or for the C70 W221 double mutant.

In this work, we mutated all of the amino acid residues plausibly involved in the catalytic activity of *E. coli* MAP to investigate the structural and functional relationships of the enzyme. Surprisingly, substitution of any amino acid residue that participates in cobalt ion binding resulted in an enzyme almost completely devoid of MAP activity. In the best case (H171L), the mutant had only 6% of wild-type activity. The residual activity may be due in part to wild-type enzymes from host cells. Although we tried to use the same amount of cell culture to isolate MAP from host cells, and negligible activity was detected in the corresponding chromatographic fractions, the possibility of contamination from the host cells cannot be ruled out.

Analysis of cobalt content revealed a complete loss of the metal ion in single mutants carrying a substitution at the metal-binding site. The cobalt/protein ratio was determined to be 1.6 for wild-type MAP. This value dropped to less than 0.1 atom per molecule for the MAP mutants. According to the X-ray crystallographic model, *E. coli* MAP displays an internal pseudo-twofold symmetry. The two cobalt ions accept ligands from each half of the enzyme (14). The side chains of Asp108 and Glu235 interact with both cobalt ions, whereas Asp97, His171, and Glu204 react with only one of them. However, the consequence of mutating any one of these residues is the same, i.e., loss of enzyme activity and both metal ions. This result demonstrates clearly that all amino acid residues at the metal-binding site are essential for the integrity of both metal coordination and enzyme activity.

A recent study showed that a MAP1 mutant from *S. cerevisiae* carrying an Asp219 substitution (corresponding to Asp97 in *E. coli* MAP) has a fivefold decrease in enzymatic activity with Met-Gly-Met-Met as a substrate (8). In addition, more than 50% of the cobalt ions were retained in the mutant enzyme compared to the wild type. A much more drastic result occurred for the *E. coli* MAP mutated at the corresponding position. Mutation at Asp97 of *E. coli* MAP caused complete loss of both enzyme activity and cobalt binding. This difference may be due in part to the variation of the replaced amino acid residue; Asp97 was changed to Ala in *E. coli* MAP while Asn was substituted for Asp219 in yeast MAP1. Assuming there are minimal changes in the backbone structure, the $O^{\delta 1}$ of the substituted Asn can still interact with the cobalt ion in yeast MAP1. This hypothesis is supported by MAP sequence alignment data. The *B. subtilis* MAP has a Gln at a position corresponding to Glu235 of *E. coli* MAP. Glu235 is a cobalt-binding residue and is conserved among other species (13). This variation does not cause a loss of MAP activity in *B. subtilis*. However, the possibility that *E. coli* and yeast MAPs are structurally distinct and that the corresponding mutants differ in cobalt-binding affinity cannot be ruled out.

The amino acid residues (Cys59, Cys70, Tyr62, Tyr65, Phe177, and Trp221) of *E. coli* MAP that plausibly are involved in substrate binding were also mutated. Conservative substitutions were carried out, if possible, to retain protein integrity. According to the X-ray crystallographic model (14), only the hydroxyl group on the Tyr65 side chain can interact with the main-chain carbonyl atoms of Val69 and Gly80. The side chains of the other five residues do not interact with any other atoms. Therefore, the substitutions that occurred in this work should not alter significantly the structure of the enzyme. Among the six amino acid residues, only two of them (Cys70 and Trp221) are closely associated with MAP activity. Mutation at either position resulted in a three- to fivefold drop in enzyme activity. The activity became almost undetectable when both residues were mutated. This finding indicates that the side chains of both amino acid residues are required for substrate binding. However, whether these mutations change the substrate specificity of the enzyme remains to be investigated.

Unlike the amino acid residues involved in metal binding, which are extremely conserved in all types of MAPs, variations do occur among the six putative substrate-binding residues (16). Except for the residue corresponding to F177 of *E. coli* MAP, which is conserved in all species reported so far, other residues are only partially conserved among species. Even though F177 is located more proximally to the cobalt ions (4.7 to 5.2 Å) among these six residues, it does not play a role in substrate binding, as demonstrated by mutational analysis (Table 2). Interestingly, when Cys70 and Trp221 of *E. coli* MAP were aligned with those of other reported MAP sequences, both residues were found to be conserved among type I, but not type II, MAPs (16). This finding implies that type I MAPs differ in substrate binding and enzyme activities from type II MAPs. With the same peptide used as a substrate, a variation

in activity can be observed between MAP1 (type I) and MAP2 (type II) from *S. cerevisiae*. Setting the catalytic activity needed to remove the methionine from a peptide with a Met-Ala-Ser sequence at 100% for both yeast MAPs, the activity was 36 and 74% for MAP1 and MAP2, respectively, when Met-Pro-Gly was used as the substrate (5, 9). This result suggests that type I and type II MAPs may differ in the mechanism of or affinity for substrate binding, although they possess the same mode of binding cobalt ions.

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