Identification of active-site residues in *Bradyrhizobium japonicum* malonamidase E2

Hyun Min KOO, Sung-Ook CHOI, Hyun Mi KIM and Yu Sam KIM¹

Department of Biochemistry, College of Science, Bioproducts Research Center, Yonsei University, Seoul 120-749, Korea

Malonamidase (MA) E2 was previously purified and characterized from *Bradyrhizobium japonicum* USDA 110. The gene encoding this enzyme has been cloned, sequenced and expressed in *Escherichia coli*. The recombinant MAE2 was purified to homogeneity from the transformed *E. coli*. The biochemical properties of the recombinant enzyme are essentially identical to those from wild-type *B. japonicum*. A database search showed that the MAE2 protein has a high sequence similarity with the common signature sequences of the amidase family. The only exception is that the aspartic residue in these signature sequences is replaced by a glutamine residue. In order to identify amino acid residues essential for enzyme activity, a series of site-directed mutagenesis studies and steady-state kinetic experiments were performed. Gln¹⁹⁵, Ser¹⁹⁹, Cys²⁰⁷ and Lys²¹³ of the common

signature sequences were selected for site-directed mutagenesis. Among the mutants, Q195D, Q195E and S199C showed less than 0.02 % of the k_{cat} value of the wild-type enzyme, and S199A, Q195L and Q195N exhibited no detectable catalytic activities. Mutants (K213L, K213R and K213H) obtained by replacement of the only conserved basic residue, Lys²¹³, in the signature sequences, also displayed significant reductions (approx. 380-fold) in k_{cat} value, whereas C207A kept full activity. These results suggest that MAE2 may catalyse hydrolysis of malonamate by a novel catalytic mechanism, in which Gln¹⁹⁵, Ser¹⁹⁹ and Lys²¹³ are involved.

Key words: glutamine, serine, lysine, site-directed mutagenesis, steady-state kinetics.

INTRODUCTION

Three malonamidases (MAs), MAE1a, MAE1b, and MAE2, have previously been purified and characterized from *Bradyrhizobium japonicum* [1]. These enzymes are known to catalyse all or some of the following reactions: malonyl transfer to hydroxylamine; hydroxylaminolysis of malonamate; and hydrolysis of malonamate. Among them, MAE2 is highly specific for hydroxylaminolysis and hydrolysis of malonamate. Its affinity for malonamate is 30- and 70-fold higher than that for MAE1a and MAE1b respectively [1]. These enzymes were first found in *B. japonicum* bacteroids and the plant cytosol of soybean nodules. It has been proposed that they may be involved in the transport of fixed nitrogen from bacteroids to the plant cell in symbiotic nitrogen metabolism [2].

Acylamide amidohydrolases (amidases; EC 3.5.1.4) catalyse hydrolysis of carboxylic acid amides to carboxylic acids and free ammonium. These enzymes are involved in nitrogen metabolism in cells and are widely distributed in living organisms: they have been found in both prokaryotic and eukaryotic cells. Among bacterial enzymes, amidases from *Brevibacterium* sp. R312 and *Pseudomonas aeruginosa* are well characterized. Based on the results of experiments performed with inhibitors, they have been classified as sulphhydryl enzymes [3,4], which belong to an amidase signature family. This family has a highly conserved linear sequence rich in serine and glycine residues which spans nearly 50 amino acids in length (Figure 1) [5,6]. In addition to MAE2, more than 45 proteins containing the amidase signature sequence have been identified to date. They include many prokaryotic [6–12] and fungal enzymes [13–15], one avian enzyme [16], one mammalian enzyme [17] and three putative members from

Caenorhabditis elegans. Recently, Kobayashi and his co-workers showed that the active-site residues of *Rhodococcus rhodochrous* J1 amidase were Asp¹⁹¹ and Ser¹⁹⁵ rather than the generally accepted Cys²⁰³ residue [18]. The catalytic features of rat fatty acid amide hydrolase (FAAH), which is the only characterized mammalian member of the amidase family, were investigated through mutagenesis, affinity labelling and kinetic studies. Affinity labelling of FAAH with ethoxyoleoyl fluorophosphonate, a specific nucleophilic inhibitor, has demonstrated that the Ser²⁴¹ residue is the enzyme's catalytic nucleophile [19]. In spite of significant progress in expanding our knowledge of amidases, little is known about their catalytic mechanism and/or tertiary structure.

In the present study we describe the molecular cloning of the *B. japonicum* MAE2 gene and the expression of the gene in *Escherichia coli*. Our results from mutagenesis and steady-state kinetic experiments clearly demonstrate that Gln¹⁹⁵, Ser¹⁹⁹ and Lys²¹³ are essential for MAE2 catalytic activity. Identification of the active-site residues demonstrates not only the specificity of the enzyme for malonamate, but also enhances our understanding of the enzyme's reaction mechanism.

EXPERIMENTAL

Strains and plasmids

B. japonicum USDA 110 was obtained from the Genetic Engineering Research Institute (Daeduck, Korea). *E. coli* XL-1 Blue MRA (P2) and MRA (Stratagene) were used to construct the genomic library of *B. japonicum*. For subcloning, *E. coli* XL-1 Blue MRF' (Stratagene) was used as a host strain. A pCR2.1

Abbreviations used: FAAH, fatty acid amide hydrolase; IPTG, isopropyl β -D-thiogalactoside; LB, Luria–Bertani; MA, malonamidase.

¹ To whom correspondence should be addressed (e-mail yskim@bubble.yonsei.ac.kr).

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Preparation of the probe

The N-terminal amino acid sequence of MAE2 is MISLADL-QRRIETGELSPNAAI. A sense primer [5'-TGATCI(G/C)-(G/C)(C/T)T(G/C)GC(G/C)GA(C/T)(C/T)T(G/A/T/C)CA-3'] of the N-terminal amino acid sequence (MISLADLQ) and an antisense primer [5'-GGT(G/C)GGCAT(A/G)TT(G/C)GC-(G/A/T/C)GT(A/G)TC-3'] of an internal amino acid sequence (TDANMPT) were prepared to make a probe. By using the following PCR procedure and the degenerate primers, a PCR product was prepared. Reaction mixtures (100 μ l) for PCR contained 100 ng of *B. japonicum* total DNA, 1.25 mM of each primer, 2.5 units of *Taq* DNA polymerase, 2 mM of each dNTP, and 1 μ l of 10 $\times$ PCR buffer (Boehringer Mannheim). The PCR reaction was carried out in a Techne Progene programmable cycling heat block for 30 cycles of 50 s of denaturation at 95 °C, 30 s of annealing at 45 °C and 60 s of extension at 72 °C, followed by one cycle of 50 s of denaturation at 95 °C and 30 s of annealing at 45 °C, 7 min of extension at 72 °C. The 216-bp PCR product was purified from a 12% polyacrylamide gel, cloned into a pCR 2.1 vector (Invitrogen) according to the manufacturer's protocol and sequenced. The product was radiolabelled with [α -³²P]dCTP using a Prime-a Labeling system (Promega) and was subsequently used for screening the λ DNA library.

Cloning strategy and DNA sequence analysis

The λ library of *B. japonicum* total DNA was screened by plaque hybridization with the radiolabelled 216-bp PCR fragment as a probe. Positive plaques were isolated by successive plating, and a pure phage was purified using a plate lysate method. A 2-kb *Bam*HI fragment inserted into the positive λ phage DNA was subcloned into pBluescript KS II (designated pBMAE2). Appropriate restriction fragments of the DNA were prepared for sequencing. These were sequenced using the ABI PRISM[®] Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) and the ABI 310 automated sequencer (Applied Biosystems Division of Perkin-Elmer).

Construction of expression plasmid with the MAE2 gene

Since there were no suitable restriction sites upstream of the presumed ATG initiation codon, it was necessary to modify the sequence upstream of the ATG codon in order for expression to occur. Modification was achieved by using PCR with the recombinant plasmid (pBMAE2) as a template and the following two primers. Primer 1, GCGGATCCAAGAAGGGTATGAG-CCatgatctcactgccga, contained 4 segments: (1) a *Bam*HI recognition site (bold); (2) a ribosome-binding site (italicized); (3) a TGA stop codon (underlined); and (4) the MAE2 structural gene that begins with the ATG start codon (lower-case letters). Primer 2, GCGGATCCgtgcatgatcagtcg, contained 16 nucleotides of the gene complementary to nucleotides 1293–1308 (lower case letters) and a *Bam*HI recognition site (bold). The plasmid designated as pMAE2 was constructed by ligation of the PCR product with pUC18/*Bam*HI. This was then used to transform *E. coli* AD494 (DE3) (Novagen, Madison, WI, U.S.A.).

Construction of recombinant plasmid for the expression of MAE2 with a His-tag

Expression vector pRSET A (Invitrogen), which is designed to express proteins containing a His-tag at the N-terminal was used in order to facilitate the enzyme's recovery and subsequent purification. The MAE2 gene was amplified with two oligonucleotides, 5'-GCGGATCCATGATCTCACTCGCCGA-3'

Table 1 Oligonucleotides used for site-directed mutagenesis

Nucleotide sequences corresponding to the mutated amino acid residues are underlined.

MAE2 gene allele	Mutagenic primer set
Q195D	CGAGCCGCCGGTATCGGTGCCACGCCAGCGG GCGCTGGGCACCGATACCGCGGCTCGGTGATC
Q195E	CGAGCCGCCGGTGAAGGTGCCAGGCCAGCGG GCGCTGGGCACCTTCACCGCGGCTCGGTGATC
Q195L	CGAGCCGCCGGTCAGGGTGCCAGGCCAGCGG GCGCTGGGCACCGTACCGCGGCTCGGTGATC
Q195N	CGAGCCGCCGGTAAATGTGCCAGGCCAGCGG GCGCTGGGCACCATACCGCGGCTCGGTGATC
S199A	GGGCCGGATACCGCGGCGCGGTCTGGGTGCC CAGACCGCGCGCGGTGATCCGCCCGCGCGC
S199C	GGGCCGGATCACTGCGCGCGCGGTCTGGGTGCC CAGACCGCGCGCGCATGATCCGCCCGCGCGC
C207A	CCGCCGGCTATCGCGGACGCCGCTGGGTGCC CGCGCGCGTCCCGGATAGCGCGGCGCGGAT
K213L	GCGGAACGACGCGGATCGCGCGCGTCCCGCA ACGGCCGCGCATCTCGCGTCTCCGCATGCTG
K213H	GCGGAACGACGCGGATCGCGCGCGTCCCGCA ACGGCCGCGCATCCATCCGCTCTCCGCATGCTG
K213R	GCGGAACGACGCGGATCGCGCGCGTCCCGCA ACGGCCGCGCATCGCGCGTCTCCGCATGCTG

and 5'-GCGGATCCGTGCGATGATCAGTGC-3' (the *Bam*HI recognition site is shown in bold). For PCR amplification, 50 ng of pMAE2 was used as a template in a 50 μ l reaction system containing 0.2 mM of each dNTP, 1.5 mM MgCl₂, 25 pmol of each primer, 1 $\times$ *Pfu* reaction buffer (Stratagene) and 2.5 units of *Pfu* DNA polymerase (Stratagene). The PCR reaction was carried out in a Techne Progene programmable cycling heat block for 25 cycles of 50 s of denaturation at 96 °C, 50 s of annealing at 52 °C and 3 min 30 s of extension at 72 °C, followed by one cycle of 50 s of denaturation at 96 °C, 50 s of annealing at 52 °C and 10 min of extension at 72 °C. The amplified product was digested with *Bam*HI and was subcloned into the *Bam*HI site of the expression vector pRSET A. The resulting plasmid was named pHMAE2.

Site-directed mutagenesis

Appropriate pairs of mutagenic primers (see Table 1) were synthesized and used to generate the mutated MAE2 by PCR, according to the method of QuickChange site-directed mutagenesis from Stratagene. Here, the pHMAE2 was used as the template for *Pfu* DNA polymerase (Stratagene). Following PCR, the wild-type parental plasmid remaining in the PCR product was selectively digested by the *Dpn*I restriction enzyme (Boehringer Mannheim), and the resultant mixture was used to transform chemically competent *E. coli* XL-1 Blue MRF' (Stratagene). The desired mutants were sequenced to identify each mutation and confirm their fidelity.

Expression and purification of wild-type MAE2

MAE2 was expressed in *E. coli* AD494 (DE3) (Novagen) transformed with pMAE2. *E. coli* RNA polymerase recognized a Lac promoter on pUC18 that was used as a vector for subcloning. The transformed *E. coli* were cultured for 12 h in 3 ml of Luria-Bertani (LB) medium containing ampicillin (50 μ g/ml), and then transferred into 200 ml of the same medium. The culture was incubated at 37 °C for 3 h and isopropyl 1-thio- β -D-galactoside (IPTG) (1 mM final conc.) was added, followed

by incubating the culture at 37 °C for 16 h. The cells were harvested and resuspended in 20 mM Tris/HCl (pH 7.4)/15% (v/v) glycerol. The preparation of crude extract followed by Affi-Gel Blue column chromatography were performed by the method described previously [1]. The adsorbed proteins were sequentially eluted by the application of a 400-ml linear gradient of NaCl (0–2 M) in the same buffer and a 300-ml stepwise gradient of 2 M NaCl in 20 mM Tris/HCl (pH 7.4). Fractions were collected and assayed for MAE2 activity. Fractions containing the enzyme activity were pooled. ZnSO₄ was added to the enzyme solution to give a final concentration of 0.5 mM. This solution was subsequently applied to a Phenyl-Sepharose CL-4B column, equilibrated with 20 mM Tris/HCl (pH 7.4) containing 2 M NaCl and 2 mM ZnSO₄. After loading was completed, the unadsorbed materials were washed with 100 ml of the same Tris buffer containing 2 M NaCl and 2 mM EDTA. MAE2 was eluted by the application of a 300-ml linear descending gradient (2–0 M) of NaCl in 20 mM Tris/HCl (pH 7.4) containing 2 mM EDTA. Fractions containing the enzyme activity were pooled and dialysed twice against 2 litres of the same Tris buffer. The dialysed solution was loaded on to Red-120 agarose, pre-equilibrated with the same buffer. As soon as the loading was completed, the adsorbed proteins were eluted by the application of a linear gradient of NaCl (0–2 M) in the same buffer. MAE2 was eluted at 1.7–1.9 M NaCl. The MAE2 solution was dialysed against the same Tris buffer, stored at 4 °C, and used for all subsequent experiments.

Expression and purification of MAE2 and mutants with a His-tag

The expression and purification of MAE2 with a His-tag were accomplished according to the manufacturer's instructions (Novagen). *E. coli* BL21 (DE3) pLysS (Novagen) transformed with pHMAE2 were cultured for 12 h in 3 ml of LB medium containing ampicillin (50 µg/ml), and were then transferred into 200 ml of the same medium. The culture was incubated at 37 °C for 2 h and IPTG (0.5 mM final conc.) was added. This was followed by incubating the culture at 25 °C for 20 h. The cells were harvested, resuspended in 8 ml of binding buffer [20 mM Tris/HCl (pH 7.9)/5 mM imidazole/500 mM NaCl] and sonicated. The cell lysates were put on to a His-Bind resin column. The protein bound to the matrix was eluted with elution buffer [20 mM Tris/HCl (pH 7.9)/400 mM imidazole/500 mM NaCl]. This protein was dialysed against 2 litres of 10 mM Tris/HCl (pH 8.0), stored at 4 °C, and used for all subsequent experiments. Each of the mutants was also expressed and purified under the same conditions.

CD spectroscopy

CD spectroscopy was used to compare the overall secondary structure of wild-type and selected mutant proteins. Measurements were recorded at room temperature from 190–250 nm under nitrogen using a JASCO J-720 spectropolarimeter with a 1 mm cell and 0.2 nm wavelength increments. Each spectrum was normalized for protein concentration and the observed ellipticity (θ) was background-corrected against the spectrum obtained for the dialysis buffer [10 mM Tris/HCl (pH 8.0)].

Enzyme assays and kinetic experiments

MAE2 activity was measured by the method described previously [1]. The method was based on hydroxylaminolysis of malonamate:

Reaction mixtures (500 µl) contain 100 mM Tris/HCl buffer (pH 8.0), 10 mM malonamate, neutralized 20 mM NH₂OH and enzyme. After incubation for 30 min at 37 °C, the malonohydroxamate formed was determined by the same method described previously for malonyl-CoA synthetase [21]. The K_m and k_{cat} values were measured as described above with various substrate concentrations (0.625–20 mM). The enzyme concentrations used in this experiment were 0.042 µM for the wild-type enzyme and 2.1 µM for each mutant. The rate of malonohydroxamate formation increased with increasing substrate concentration; demonstrating typical Michaelis–Menten saturation kinetic behaviour. The double-reciprocal plots were linear, and from these plots the kinetic parameters were determined. The error values reported reflect the standard deviation of at least three independent trials.

RESULTS

Cloning and nucleotide sequence of the MAE2 gene from *B. japonicum* USDA 110

Degenerate oligonucleotides corresponding to the amino acid sequences of the N-terminal region of the protein and of internal peptides were synthesized and used to clone a 216-bp DNA fragment from *B. japonicum* total DNA. This partial clone was then used as a probe to screen a *B. japonicum* total DNA library for full-length clones. Clones containing the entire gene were isolated. The DNA prepared from these positive clones was subjected to restriction analysis and Southern-blot hybridization with the probe, which indicated the location of the MAE2 gene. A 2-kb *Bam*HI DNA fragment that contained the entire gene was isolated and subcloned into pBluescript KS II, and the clone thus obtained was designated as pBMAE2. The nucleotide sequence of the MAE2 gene was previously deposited at the GenBank database under the accession number AF012735. The MAE2 gene encodes a polypeptide of 432 amino acids with a calculated molecular mass of 45635 Da. The N-terminal and three tryptic peptide sequences for MAE2 of *B. japonicum*, MISLADLQRRIETGELSPNAAI, DPTDTANMPT, WQPR-HD, and AGASVQAIDL, match the nucleotide sequence in the MAE2 gene, thus confirming that the gene encodes MAE2.

A database search showed that the MAE2 protein sequence shares close homology to several amidase enzymes from organisms as diverse as *Methanobacterium thermoautotrophicum* [22], *Pseudomonas chlororaphis* [23], *Rhodococcus* sp. [6,8], *R. rhodochrous* [7], *Saccharomyces cerevisiae* [24], *C. elegans* [25], *Flavobacterium* [9], *B. japonicum* [26], *Comamonas acidovorans* [27] and rat [17]. These amidases collectively make up an enzyme family whose members all share a common signature sequence (Figure 1).

Purification and characterization of MAE2 expressed in *E. coli*

When *E. coli* harbouring pMAE2 were cultured for 12 h at 37 °C in LB medium in the presence of 1 mM IPTG, the MAE2 activity in the supernatant of the sonicated cell-free extract of the transformant was 0.67 µmol/min per mg of protein, which was 3.4-fold higher than that in *B. japonicum* USDA 110 (with malonamate as the substrate). The MAE2 was easily purified as described in the Experimental section. The enzyme from the Red-120 agarose gel was homogeneous on SDS/PAGE (results not shown) after approx. 386-fold purification with a recovery of approx. 43% (Table 2). The specific activity of the purified recombinant enzyme was 251 µmol/min per mg of protein, for malonamate, at pH 8.0 and 37 °C, corresponding well to the reported value of 269 µmol/min per mg of protein for the protein

Table 2 Purification of MAE2 from transformed *E. coli* harboring pMAE2

Starting material: 8 g bacteria (wet mass). Enzyme activity was measured as described in the Experimental section.

Purification	Volume (ml)	Total protein (mg)	Total activity ($\mu\text{mol}/\text{min}$)	Specific activity ($\mu\text{mol}/\text{min}$ per mg)	Recovery	
					(%)	Fold
Crude extract	60	1432	954	0.67	100	1
Affi-Gel Blue	260	78	667	8.5	70	12.7
Phenyl-Sepharose	51	12.8	532	41.6	56	62
Red-120 agarose	53	1.58	410	259	43	386

purified from *B. japonicum*. The k_{cat} values of the recombinant enzyme were essentially identical to the parental enzyme from *B. japonicum* ($2.2 \times 10^3 \text{ s}^{-1}$ and $2.1 \times 10^3 \text{ s}^{-1}$ respectively). The substrate specificity of the enzyme was examined by using other substrate analogues (malonate, succinate, acetate, succinamate, malonamide and acetamide). However, these analogues could not be used as substrates, instead of malonamate, showing that the enzyme is highly specific for malonamate. These results indicate that the properties such as specific activity, molecular mass, substrate specificity and kinetic properties of the enzyme expressed in *E. coli* were more or less identical to those of the parental MAE2.

Identification of putative active-site residues

The amino acid sequence of MAE2 identified this enzyme as a member of the amidase family, as mentioned above. This enzyme family is defined by a highly conserved linear sequence which spans nearly 50 amino acids in length (Figure 1) [5,6]. MAE2 shows a sequence similarity with the common signature sequence except that, interestingly, a glutamine residue is located at the position corresponding to the conserved aspartic residue in the common signature sequences (Figure 1). So far, members of the amidase family have been generally classified as one of the sulphhydryl enzymes [3,4]. Recently, two active-site amino acid residues, aspartic acid and serine, have been identified within these common signature sequences of the *R. rhodochrous* J1 amidase [18]. It was suggested that Asp¹⁹¹ of the *Rhodococcus* amidase may function as a general base drawing the proton from the Ser¹⁹⁵ C³ hydroxy group, allowing the serine to act as a nucleophile attacking the carbonyl group of the amide junction within the substrate. More recently, the corresponding serine (residue 199 in MAE2 numbering) in the FAAH sequence was identified as a catalytic nucleophile through mutagenesis and affinity labelling studies [19]. Considering the above results, the glutamine residue together with the serine residue was expected to be a candidate for the active-site amino acid residue of MAE2. We thus selected Gln¹⁹⁵ and Ser¹⁹⁹, as well as Cys²⁰⁷, for mutagenesis. To test the possibility that a highly conserved basic residue served as the catalytic base of MAE2, we also selected Lys²¹³, which is the only conserved basic residue in the signature sequence (Figure 1).

Kinetic properties of MAE2 mutants

In order to identify active-site residues essential for MAE2 function, wild-type MAE2 and its site-specific mutants were produced as His-tagged fusion proteins in *E. coli*. Ten different mutant enzymes (Q195D, Q195L, Q195N, Q195E, S199A, S199C, K213L, K213H, K213R and C207A), as well as wild-type MAE2, were prepared and their hydrolytic activities, with

Figure 2 Circular dichroism spectra of purified wild-type and mutant MAE2 enzymes

The spectra were recorded in 10 mM Tris/HCl buffer, pH 8.0, in the far-UV region between 190 and 250 nm at 25 °C. Spectra of protein samples (approximately 0.3 mg/ml) were measured in a 300 μl quartz cell of 1 mm path length. The lines A–E represent wild-type, Q195E, S199A, K213H and C207A respectively. The CD spectra of the Q195D, Q195L, Q195N, S199C, K213L and K213R mutants were within error of the spectra shown but were omitted for clarity.

malonamate as substrate, were determined. The mutant enzymes were successfully produced in *E. coli* BL21 (DE3) pLysS and were purified under the same conditions as the wild-type MAE2. Their CD spectra in the far UV region were almost identical to that of the wild-type enzyme with a His-tag (Figure 2), indicating that no major changes occurred in the overall conformation of the mutant enzymes. Specific activities of S199A, Q195L and Q195N mutant enzymes were below the detection level ($\leq 0.01\%$ of the original activity), even when large amounts of the enzymes were used in the reaction for 16 h. The specific activity of the wild-type enzyme with a His-tag was similar to that of the parental enzyme from *B. japonicum* ($228 \mu\text{mol}/\text{min}$ per mg of protein and $269 \mu\text{mol}/\text{min}$ per mg of protein respectively). The k_{cat} values of the two enzymes were essentially identical ($2.2 \times 10^3 \text{ s}^{-1}$ and $2.1 \times 10^3 \text{ s}^{-1}$ respectively), suggesting that the His-tag had no effect on the Michaelis–Menten parameters.

The steady-state kinetic analysis of the mutant enzymes showed that mutations of the three residues, Gln¹⁹⁵, Ser¹⁹⁹ and Lys²¹³, resulted in MAE2 enzymes bearing significant catalytic defects (Table 3). The k_{cat} value for malonohydroxamate formation, which was 2200 s^{-1} in the wild-type, was decreased by approx. 7×10^3 -fold in the Q195D and Q195E mutants, 1.2×10^4 -fold in the S199C mutant and approx. 380-fold in the K213 mutants. The K_m of the Q195E mutant was 3-fold higher than that of the wild-type enzyme. The difference occurred due to the change of the ionization state caused by the substitution. Differences in the K_m values of other mutants were insignificant compared to that of the wild-type. This implied that the substitutions had no influence on substrate binding. The C207A mutant exhibited catalytic properties similar to those of wild-type MAE2 (Table 3). The results indicate that Ser¹⁹⁹, Lys²¹³ and Gln¹⁹⁵ are crucial to the catalytic activity of MAE2, whereas Cys²⁰⁷ is not. This is in agreement with the finding that the purified wild-type MAE2

Table 3 Kinetic properties of MAE2 mutants

Assay conditions were as described in the Experimental section. Values represent means $\pm$ S.D. ($n \geq 3$).

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Wild-type	3.76 ± 0.4	2200 ± 160	585000
Q195E	12.1 ± 1.1	0.32 ± 0.04	26.4
Q195D	1.81 ± 0.2	0.30 ± 0.03	166
S199C	6.94 ± 0.7	0.18 ± 0.01	25.9
C207A	4.21 ± 0.1	2400 ± 270	570000
K213L	2.28 ± 0.2	5.8 ± 1.1	2540
K213H	2.03 ± 0.1	5.8 ± 0.9	2860
K213R	1.8 ± 0.4	5.7 ± 0.4	3170

shows a strong resistance to thiol reagents, such as *N*-ethylmaleimide and iodoacetamide (results not shown), and with the finding that Cys²⁰⁷ is not completely conserved in every member of the amidase family (Figure 1).

DISCUSSION

We have investigated the cloning and sequencing of the MAE2 gene from *B. japonicum*, which allow the comparison of the amino acid sequence derived from its DNA with those of the amidase signature family reported so far. MAE2 shows sequence similarity with the common signature sequence of amidases. It is interesting to note that in MAE2 a glutamine residue occupies the position of the catalytic aspartic residue found in the common signature sequences (Figure 1). The present study also describes site-directed mutagenesis experiments on the MAE2 from *B. japonicum*.

MA was previously known to form a covalent intermediate during catalysis. The isolation of [¹⁴C]malonyl-enzyme by gel-filtration and isotope-exchange experiments with [¹⁸O]malonate indicated that the enzyme operates by an acyl-enzyme mechanism [1]. MAE2 is not inactivated by the serine-directed inhibitor, PMSF (results not shown). Although this result seems to be inconsistent with the crucial role of Ser¹⁹⁹ as revealed by the site-directed mutagenesis experiments, such apparent inconsistency is known in several serine proteases. For example, leader peptidase, which removes signal peptides from exported proteins, is a serine protease, yet it is not sensitive to diisopropyl fluorophosphate ('DIFP') or PMSF [28]. It is also interesting to note that the S199C mutant showed some enzyme activity (approx. 0.025% of the original activity), demonstrating indirectly the importance of Ser¹⁹⁹ as a nucleophile. In support of this notion, the corresponding serine in rat FAAH was identified as the enzyme's catalytic nucleophile [19].

The amide bond (CO-NH₂) in the amide substrate is structurally not too far removed from a peptide bond (CO-NH-). Recently, Kobayashi and his co-workers proposed an evolutionary relationship between amide-bond cleaving enzymes (including amidases) and peptide-bond-cleaving enzymes (including aspartic proteinases) [18]. However, MAE2 deviates from the amidase signature sequence in that a conserved aspartic residue is replaced with a glutamine residue (residue 195 in MAE2 numbering). We should therefore consider the possibility that the catalytic mechanism of the enzyme might resemble that of serine proteases. The standard mechanism for proteases, especially serine proteases, which has evolved both by convergent and divergent evolution [29], involves a catalytic triad. The

classical catalytic triad consists of a serine nucleophile, a histidine that acts as a general base to activate the hydroxy group of the serine, and an aspartate, which stabilizes the partial charge assumed by the histidine [30]. In the case of rat FAAH, the FAAH mutants H184Q, H358A and H449A, which involve the only three conserved histidine residues among rat, mouse and human FAAHs, retained almost full activity, suggesting that a histidine base does not participate in FAAH's catalytic mechanism [19]. As shown in Figure 1 there are no histidine or aspartic residues in the common signature sequence of MAE2, whereas there are both a highly conserved lysine residue and a serine residue. Moreover, we found that there are no conserved histidine or aspartic residues when the amino acid sequence of MAE2 is aligned with all previously reported members of the amidase family (results not shown). These results suggest that MAE2 does not contain a Ser-His-Asp catalytic triad commonly found in other serine hydrolytic enzymes. Among serine hydrolases, class A β -lactamases [31], the type I signal peptidases [32] and the Lex repressor [33] have been found to utilize a non-histidine base in catalysis. In these unusual cases, a lysine residue is thought to function as the general base to assist in nucleophilic attack by the serine hydroxy group. MAE2 has Lys²¹³ that is highly conserved in the signature sequence of the amidase family. It is very interesting to note that when lysine is replaced by other amino acids in the three mutants we have examined, namely K213L, K213R and K213E, the mutants exhibit significant catalytic defects suggesting the importance of Lys²¹³ in enzymic activity. Although the specific role of the lysine residue in MAE2 catalysis has not been established, it is most likely that Lys²¹³ functions as a base in the activation of MAE2's serine nucleophile. Further characterization of MAE2's kinetic mechanism and high-resolution structural studies may elucidate the potential role of the lysine residue in MAE2's catalytic mechanism.

Mutation of Gln¹⁹⁵ to glutamic acid, aspartic acid, leucine or asparagine in MAE2 revealed that it plays a significant role in catalysis. The reaction of amidases is thought to proceed via an acyl-enzyme intermediate like an oxyanion derivative of the peptide in serine protease catalysis [17,34]. We suggest that Gln¹⁹⁵ may participate in the stabilization of the transition-state complex, by forming a hydrogen-bond to the oxyanion. A similar role for asparagine has been proposed in the catalytic mechanism of subtilisin from *Bacillus amyloliquefaciens* [34] and penicillin acylase from *E. coli* [35]. In the case of subtilisin, preventing formation of hydrogen-bonds in the oxyanion hole by mutating asparagine to alanine decreases its reaction rate by more than 1000-fold, which emphasizes the importance of this residue in hydrolysis.

In summary, we have sequenced, cloned and expressed the *B. japonicum* MAE2 in *E. coli*, and the Gln¹⁹⁵, Ser¹⁹⁹ and Lys²¹³ in the signature sequences were identified to be part of the active-site of the enzyme. This is the first report that an amidase having the common signature sequence catalyses hydrolysis of an amide by a novel catalytic mechanism in which serine, glutamine and lysine are involved. This finding will be instrumental for understanding the catalytic action of the amidase family.

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