Arg-158 Is Critical in Both Binding the Substrate and Stabilizing the Transition-state Oxyanion for the Enzymatic Reaction of Malonamidase E2

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Malonamidase E2 (MAE2) from Bradyrhizobium japonicum is an enzyme that hydrolyzes malonamate to malonate and has a Ser-cis-Ser-Lys catalytic triad at the active site. The crystal structures of wild type and mutant MAE2 exhibited that the guanido group of Arg-158 could be involved in the binding of malonamate in which the negative charge of the carboxyl group could destabilize a negatively charged transition-state oxyanion in the enzymatic reaction. In an attempt to elucidate the specific roles of Arg-158, site-directed mutants, R158Q, R158E, and R158K, were prepared (see Table 1). The crystal structure of R158Q determined at 2.2 Å resolution showed that the guanido group of Arg-158 was important for the substrate binding with the marginal structural change upon the mutation. The $k_{cat}$ value of R158Q significantly decreased by over 1500-fold and the catalytic activity of R158E could not be detected. The $k_{cat}$ value of R158K was similar to that of the wild type with the $K_m$ value drastically increased by 100-fold, suggesting that Lys-158 of R158K can stabilize the negative charge of the carboxylate in the substrate to some extent and contribute to the stabilization of the transition-state oxyanion, but a single amine group of Lys-158 in R158K could not precisely anchor the carboxyl group of malonamate compared with the guanido group of Arg-158. Our kinetic and structural evidences demonstrate that Arg-158 in MAE2 should be critical to both binding the substrate and stabilizing the transition-state oxyanion for the catalytic reaction of MAE2.

Serine-nucleophile hydrolase is one of the most widespread enzymes in biological system and includes proteases, esterases, and lipases of which the catalytic triads or dyads play a critical role in the enzymatic catalysis (1). In most cases, enzymatic hydrolysis involves the nucleophilic attack on the electrophilic carbonyl carbon in the substrate, resulting in the formation of an oxyanion. The oxyanion can be recognized and stabilized by the oxyanion hole for the rate enhancement of the enzymatic reaction (2). Consequently, the stabilization of the transition-state oxyanion is very important for the efficient catalysis in the serine-nucleophile hydrolase.

An enzyme family, designated as amidase signature (AS)2 enzymes, utilizes a novel Ser-cis-Ser-Lys catalytic triad that is distinctly different from the classical catalytic triad or its variant (3–5). The unique nomenclature of AS is attributed to the AS sequence including the conserved sequence of about 130 amino acids rich in glycine and serine (Fig. 1A) (6, 7). The AS enzyme family includes over 200 different enzymes in many different organisms. The major role of AS enzymes has been known to be the hydrolysis of the amide bond. The enzymatic reactions of AS enzymes are involved in many critical biological functions such as the catabolism of neuromodulatory fatty acid amide by fatty acid amide hydrolase (8–11), the formation of Gln-tRNA$^\text{Gln}$ by aminotransferase (12, 13), the formation of indole-3-acetic acid, which plays a role as a key regulator in plant development (14), and the metabolic turnover of carbon-nitrogen compounds (6, 15). The amidase is also involved in the industrial scale production of acrylamide and nicotinamide (16). It has been suggested that most AS family enzymes utilize the Ser-cis-Ser-Lys catalytic triad (Fig. 1B) in which Ser-155 plays an essential role as a nucleophile to attack the electrophilic carbonyl carbon of the substrate, with the subsequent formation of a tetrahedral intermediate containing a very unstable oxyanion (4, 17, 18).

Malonamidase E2 (MAE2) from B. japonicum, one of AS enzymes, catalyzes the hydrolysis of malonamate to malonate (3, 17). MAE2 was supposed to utilize four backbone-NH groups, which can stabilize the negatively charged transition-state oxyanion by forming hydrogen bonds (3). The transition-state oxyanion originating from the malonamate substrate can be inevitably destabilized due to neighboring negative charge of a carboxylate of the substrate, leading to the destabilization of the transition state and the significant decrease in catalytic activity. Crystal structure of S131A in complex with malonamate shows that the Ser-cis-Ser-Lys catalytic triad is recognized and stabilized by the oxyanion hole for the rate enhancement of the enzymatic reaction (2). Consequently, the stabilization of the transition-state oxyanion is very important for the efficient catalysis in the serine-nucleophile hydrolase.

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The atomic coordinates and structure factors (code 1OBK) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The abbreviations used are: AS, amidase signature; MAE2, malonamidase E2 from Bradyrhizobium japonicum; DTT, dithiothreitol; pMAE2, pUC18 vector carrying the wild type MAE2 gene; WT, wild type; PPS, pyrophospho-serine; r.m.s.d., root mean square deviation.

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**TABLE 1**

Kinetic parameters of WT and mutant MAE2s

| Enzyme | $k_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}/K_m$ | Relative $k_{\text{cat}}$ | Relative $K_m$ |
|--------|------------------|-------|---------------------|--------------------------|----------------|
|        | s$^{-1}$ | mM | s$^{-1}$ |                           |                |
| Wild type | 1844 ± 178 | 2.96 ± 1.35 | 6.2 × 10$^5$ | 1.000 | 1.000 |
| R158K | 1657 ± 94.6 | 297 ± 73.6 | 5.6 × 10$^4$ | 0.898 | 100.4 |
| R158Q | 1.16 ± 0.08 | 3.26 ± 0.50 | 3.5 × 10$^4$ | 6.3 × 10$^{-4}$ | 1.101 |
| R158E | ND$^a$ | ND$^a$ | ND$^a$ | ND$^a$ | ND$^a$ |

$^a$ ND, Value relative to those of wild type.

Malonam suggested that the guanido group of Arg-158 could form hydrogen bonds with the carboxyl group of malonamate to assist the binding of the substrate to the active site of the enzyme (17). Arg-158 is highly conserved like those residues in the Ser-cis-Ser-Lys catalytic triad among A5 family enzymes (Fig. 1A). We postulated that those hydrogen bonds mediated by the guanido group of Arg-158 can play key roles not only in enhancing the binding affinity of the substrate but also in stabilizing the oxyanion intermediate by neutralizing the negative charge of the malonamate’s carboxyl group during the process of the catalytic reaction.

To identify the specific roles of the guanido group in Arg-158 in the substrate binding and in the stabilization of transition-state oxyanion, we prepared a series of mutant enzymes of MAE2, R158K, R158Q, and R158E. Steady-state kinetic analyses clearly suggest that the guanido group of Arg-158 not only be critical to affinity of the substrate but also contribute to the stabilization of the negatively charged oxyanion by interacting electrostatically with the negatively charged carboxylate of the substrate. The crystal structure of R158Q determined at 2.2 Å resolution and CD spectra of the mutant MAE2s indicated that little significant structural change was made by the R158Q mutation of MAE2 and that the mutational effect could be caused mostly by the substitution of Arg-158 without any noticeable structural perturbation. Our kinetic and structural data demonstrated that Arg-158 in MAE2 is critical not only to the binding affinity of the substrate but also to the stabilization of the transition-state oxyanion.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**—Chemicals for buffer solutions and red-120-agarose were from Sigma. Oligonucleotides were obtained from Genotech, Korea. Mono Q ion exchange and Superdex-75 gel filtration columns were obtained from GE Healthcare, pMAE2, pUC18 vector carrying the wild type MAE2 gene was described previously (19). Malonamic acid was prepared by the method described previously (20).

**Site-directed Mutagenesis**—All the mutageneses were carried out using the QuikChange site-directed mutagenesis kit (Stratagene) according to the procedure provided by the supplier. pMAE2 was used for the mutagenesis with two primers for each mutant: R158Q, 5'-GGCGGCTGCTGATTCAAGCCCGCCG- GCCTATTGC-3' and 5'-GCAAATAGGCGCCGCTGCTGCTG- ATACCAGGCGCCGCC-3'; R158K, 5'-GGCGGCTGCTGCTGCTGCTG- ATACCAGGCGCCGCC-3'; R158E, 5'-GGCGGCTGCTGCTGCTGCTG- ATACCAGGCGCCGCC-3'; R158F, 5'-GGCGGCTGCTGCTGCTGCTG- ATACCAGGCGCCGCC-3'; R158W, 5'-GGCGGCTGCTGCTGCTGCTG- ATACCAGGCGCCGCC-3'; R158Y, 5'-GGCGGCTGCTGCTGCTGCTG- ATACCAGGCGCCGCC-3'.

**Steady-state Kinetic Analysis**—Catalytic activities of the wild type (WT) and mutant enzymes were determined by absorption spectrophotometry according to the method described previously (20). The enzymatic reaction for kinetic analysis was started by adding the enzyme to the assay mixture adjusted to 500 μl, containing various concentrations of malonamate (4, 6, 10, 12, 14, or 16 mM), 20 mM NH$_2$OH, and 0.1 M Tris-HCl, pH 8.0. The reaction mixture was incubated at 37 °C for 30 min, and the reaction was stopped by adding 750 μl of the coloring reagent solution containing 10% trichloroacetic acid, 0.66 N HCl, and 15% FeCl$_3$.

**Crystallization and Structure Determination**—Crystals of R158Q were grown in a solution containing 20% polyethylene-glycol 1000 and 0.1 M Tris-HCl, pH 7.0, by the hanging drop method of vapor diffusion at 25 °C. The crystal was flash-cooled in the stream of nitrogen gas (100 K) prior to x-ray data collection. All data were obtained by use of a rotating anode x-ray generator Rigaku RU300 equipped with focusing x-ray mirrors, operated at 50 kV/100 mA, and the R-AXIS IV$^{2\text{nd}}$ area detector (Molecular Structure Corp.). The data were integrated with
DENZO and scaled with SCALEPACK (21). The crystal belongs to the space group \(P_2_1_2_1_2\), with cell dimensions of \(a = 103.88\), \(b = 95.41\), and \(c = 74.96\) Å. The structure of R158Q was determined by direct refinement of the structure of the wild type against diffraction data using the program CNS (22). The model contained the omission of a mutated residue.

CD Spectroscopic Analysis—CD spectra of proteins were obtained with a spectropolarimeter (Jasco, 715) equipped with a Peltier-type temperature controller (Jasco, PTC-348WI), using a quartz cuvette with 0.2-cm path length. 3 \(\mu\)M wild type or mutant enzymes was preincubated at 25 °C in a buffer containing 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 1 mM DTT. Scans were collected at 0.1-nm intervals with 1-nm bandwidth and accumulated three to five times. Each spectrum was corrected by subtracting the spectrum of the buffer solution and smoothened by use of a software program provided by the manufacturer of the spectropolarimeter.

RESULTS AND DISCUSSION

Structural Analysis of the Active Site Environment of MAE2—Analyses of crystal structures of native and mutant MAE2s revealed that Arg-158 could play crucial roles for the stabilization of the transition-state oxyanion and the recognition of the substrate. Previously we determined the crystal structure of MAE2, in which the serine nucleophile Ser-155 was covalently modified to pyrophosphoserine (PPS) to mimic the tetrahedral oxyanion intermediate during the enzyme reaction (3). The structural analysis of the modified MAE2 showed that the oxygen atom O-6 corresponding to the oxyanion in the putative transition state is 3.15 Å apart from the oxygen atom O-1 (Fig. 2). O-1 of PPS can destabilize the oxyanion O-6 due to the electrostatic repulsion between two oxygen atoms upon the generation of the oxyanion. Hence, the negative charge of the carboxylate oxygen can critically destabilize the oxyanion in the transition state. The crystal structure of MAE2 in complex with malonate showed that the guanido group of Arg-158 in MAE2 could interact with the substrate through the formation of hydrogen bonds between the positive charge of the guanido group and the negative charge of the carboxyl group (Fig. 3A). The crystal structure of S131A in complex with malonate also showed that the carboxyl group of mal-
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FIGURE 2. Region of catalytic triad including the oxyanion hole in MAE2. A, stereo view of the catalytic triad and their neighboring residues. Ser-155, which plays a role as a nucleophile, is covalently modified with phenylphosphorodiamidate resulting in the formation of PPS (Protein Data Bank code 1O9O). The backbone NH groups of Thr-152, Gly-153, Gly-154, and Ser-155 constitute an oxyanion hole, which can stabilize an oxyanion, corresponding to oxygen 6 of PPS, by forming four hydrogen bonds. The dashed lines indicate putative hydrogen bonds. O1 and O6 represent oxygen 1 and oxygen 6 of PPS, respectively. B, the close-up view of Arg-158 and PPS is drawn in another angle to clearly show their interactions. Oxygen 6 (O6) corresponding to the oxyanion in the enzyme reaction is adjacent to oxygen 1 (O1) with a distance of 3.15 Å.

FIGURE 3. Stereo view of MAE2 in complex with malonate or malonamate. A, the guanido group of Arg-158 can form hydrogen bonds with the carboxyl group of a reaction product, malonate in WT (Protein Data Bank code 1OCL). B, the guanido group of Arg-158 forms hydrogen bonds with the carboxyl group of malonamate in S131A (Protein Data Bank code 1O9O). O2 and O3 represent oxygen 2 and oxygen 3 of malonamate, respectively. Oxygen 3 (O3) is considered an oxyanion during the enzymatic reaction and the distance between oxygen 2 (O2) and oxygen 3 (O3) was determined as 3.15 Å. Figs. 1–3 were drawn using a computer program (Accelrys, WebLab ViewerPro).

TABLE 2
Crystallographic data and refinement statistics for R158Q

| Characteristic                          | Value     |
|----------------------------------------|-----------|
| Resolution (Å)                         | 2.2       |
| Rmerge (%)                             | 8.2       |
| Data completeness, F > 1 σ (%)         | 93.1      |
| Rmutant (%)                            | 17.7      |
| Rfree (%)                              | 25.1      |
| No. of refined atoms                   | 7047/925  |
| Atom/water                             | 18.3      |
| Average B factor                       | 0.0049    |
| r.m.s.d. bond length (Å)               | 1.237     |
| Ramachandran plot (%)                  | 89.6      |
| Most favored regions                   | 10.3      |
| Additional allowed regions             | 0.1       |

Generously allowed regions 0.1

\*Rmerge = \frac{\sum ||I_{obs} - \langle I_{sym} \rangle||}{\sum I_{obs}} where \langle I_{sym} \rangle is the observed intensity of individual reflection, and \sum I_{sym} is average over symmetry equivalents.

\*R-factor = \frac{\sum ||F_{o} - |F_{e}|/|F_{o}| \sum F_{e}||}{\sum |F_{o}|} where |F_{o}| and |F_{e}| are the observed and calculated structure factor amplitudes, respectively.

\*Rfree was calculated with 5% of the data.

Malonamate could interact with the guanido group of Arg-158 (Fig. 3B). The negative charge of the carboxyl group in malonamate can be neutralized and stabilized by the positive charge of the guanido group in Arg-158. Similarly, the oxygen atoms, O-1 and O-2, of PPS were found to be able to form two hydrogen bonds with the guanido group of Arg-158 in the covalently modified MAE2 (Fig. 2B). The structural analyses of MAE2s indicate that Arg-158 could play two critical roles during the enzymatic reaction process by forming hydrogen bonds between its positively charged guanido group and the negatively charged carboxyl group of malonamate. Those hydrogen bonds cannot only increase the binding affinity of the substrate but also assist the formation of the transition-state oxyanion by neutralizing and stabilizing the negative charge of the carboxyl group in the malonamate.

To investigate the role of the guanido group of Arg-158 at the molecular level, the crystal structure of R158Q was determined at 2.2 Å resolution. Unfortunately, the crystals of other mutant MAE2s could not be obtained. The statistics of the crystallographic data and refinement for R158Q are summarized in Table 2. When superimposed with the structure of WT or that of S131A in complex with malonamate to compare the atomic coordinates of all the residues, the structure of R158Q was found to be nearly identical to that of WT or S131A as judged by the r.m.s.d. value of 0.36 or 0.20 Å, respectively (Fig. 4). The overall geometry of the catalytic triad and the putative oxyanion hole comprising NH groups of Thr-152, Gly-153, Gly-154, and Ser-155 in the polypeptide backbone were also found to be maintained properly despite the R158Q mutation. CD spectra of mutant MAE2s obtained between 200 and 250 nm were very similar to that of WT, indicating that no significant structural changes were made by the substitution of arginine with glutamine at the 158 position of MAE2 (Fig. 5). The crystal structure of R158Q and CD spectra of all the mutants demonstrated that
the mutations did not cause any significant structural perturbation, allowing us to investigate the role of the guanido moiety by assessing the mutational effects of Arg-158.

**Site-directed Mutagenesis of MAE2**—The replacements of Arg-158 in MAE2 with glutamate and glutamine decreased the catalytic activity dramatically by over 1500-fold, while R158K exhibited the catalytic activity a little smaller than WT (Table 1). These observations suggested that the positive charge at the position 158 be critically required to stabilize the transition-state oxyanion by neutralizing the negative charge of the substrate adjacent to the oxyanion. The R158K mutation, however, increased the $K_m$ value by 100-fold, implying that the hydrogen bonds formed between the positively charged guanido group of Arg-158 and the negatively charged carboxyl group of the substrate can be critical to the binding of the substrate. The similar $k_{cat}$ with significantly increased $K_m$ value can represent a case of the uniform binding change (23–25) in which the free energy change ($\Delta G^\ddagger_{WT}$) between the bound state of enzyme and substrate (ES$_{WT}$) and the transition state (ES$_{WT}^*$) for WT is almost similar to that (ΔG$^\ddagger_{M}$) for the mutant, while the free energy changes for binding the substrate are different between WT and mutant (Fig. 6). In other words, the mutation giving rise to the uniform binding change can cause the binding energy for the substrate to be changed since the mutation affects the initial binding step of the substrate and an enzyme. However, the mutation does not change the activation energy required to reach the transition state. Uniform binding change based on the kinetic data indicated that the R158K mutation largely decreased the binding energy for the substrate but did not change the activation energy required to reach the transition-state oxyanion. These results suggest that the positively charged amine group of Lys-158 should neutralize the negative charge of the carboxyl group of malonamate and thereby assist the stabilization of the oxyanion, but the amine group of Lys-158 could be much less efficient than the guanido group of Arg-158 for the substrate recognition.
Molecular Interaction between the Guanido Group and the Carboxyl Group—Molecular interaction between the guanido group of arginine and the carboxyl group in the substrate or the active site residues of enzyme was reported to be crucially involved in the interaction between the substrate and the enzyme (31–36). The ionized guanido group of arginine can form a planar structure due to the sp² configuration of guanido carbon atom, so that a stable planar ion pair can be generated by forming a coplanar structure between the guanido group and the negatively charged carboxyl group. In contrast to the guanido group, the amine group of lysine cannot form the planar ion pair with the carboxyl group. Based on the crystal structures of MAEs in complex with malonamate or malonate, the ion pair formed by the guanido group of Arg-158, and the carboxyl group of malonamate or malonate was found to form an ion pair with the coplanar structure in which the interaction energy for stabilization can be maximized (Fig. 7). Similar observations were made in other enzymatic reactions. In phosphatidylinositol-specific phospholipase C, the replacement of Arg-69 with lysine largely decreased the activity toward \( R_p \)-form stereoisomer of 1,2-dipalmitoyl-sn-glycero-3-phospho-1-myoinositol by 10⁴-fold, which could originate from specific interaction with the oxygen of the phosphate group in the transition state (35). The replacement of Arg-292 and Arg-386 at the active site with lysines resulted in the alteration of the enzymatic reaction and the substrate specificity of aspartate aminotransferase due to
the change of the binding modes of the substrates (36). The substitution of Arg-292 with lysine drastically increased the $K_m$ value by 81.5-fold compared with WT, of which the guanido group forms a coplanar ion pair with the carboxyl group of aspartate substrate in the WT structure. These observations also support the notion that arginine and lysine show a distinctly different property in terms of molecular recognition. Accordingly, in R158K the amine group of Lys-158 cannot efficiently anchor the carboxyl group of substrate compared with the guanido group of Arg-158, which can form stable coplanar ion pairs with the carboxyl group of malonamate.

**Stabilization of the Transition-state Oxyanion by Neutralizing the Negative Charge of the Substrate**—In MAE2 the negative charge of the substrate should be most efficiently neutralized by the formation of the ion pairs with the positively charged side chain of Arg-158, which can prevent the transition-state oxyanion from being destabilized due to the electrostatic repulsion between negative charges of the carboxylate and the oxyanion. Through the formation of ion pairs between the guanido group and the carboxyl group, their respective positive and negative charge can be stabilized electrostatically and be neutralized. In aspartate aminotransferase two active site arginines contribute the recognition of dicarboxylate, in which Arg-386 in one subunit of a dimer interacts with the $\alpha$-carboxylate of the substrate and Arg-292 in the other subunit of the dimer interacts with the side chain carboxylate (37). It was considered that the electrostatic repulsion between Arg-386 and Arg-292 favors the open conformation of the enzyme, but in the presence of the substrate the positive charges of the two arginines could be neutralized by the negative charge of the substrate, leading to the closed conformation of the enzyme (38). In case of phosphatidylinositol-specific phospholipase C it was found that the positively charged guanido group of Arg-69 could directly stabilize the negatively charged pentacovalent transition state through hydrogen bonds between the guanido group of Arg-69 and the oxygen atom of pentacovalent transition state (39). Given that the substitution of Arg-158 with lysine in MAE2 decreased the catalytic activity by only 11%, the positive charge of arginine and lysine can neutralize the negative charge of the malonamate substrate to prevent the transition-state oxyanion from being electrostatically destabilized.

The stabilization of the transition-state oxyanion in serine-nucleasephile hydrolases is important for the efficient catalysis since the overall reaction must proceed through the formation of the tetrahedral intermediate containing an oxyanion. The oxyanion was reported to be stabilized mostly by the NH groups in the peptide backbone known as the oxyanion hole. It has also been known that the side chains of the active site residues in the enzyme can further stabilize the oxyanion through direct interaction with the oxyanion by forming a hydrogen bond between the oxyanion and the hydroxyl group of serine or tyrosine (40–43). In MAE2, the stabilization of the transition-state oxyanion by neutralizing the negative charge of the substrate contrasts with the stabilization only through both the oxyanion hole and the side chains mentioned above. The stabilizing effect originates partly from indirect interaction between the oxyanion and the side chain of the amino acid apart from the oxyanion. The side chain of Arg-158 in MAE2 does not interact directly with the oxyanion but with the functional group of the substrate whose negative charge can destabilize the negatively charged oxyanion during the enzymatic reaction. The side chain such as a positively charged side chain of arginine can neutralize this negative charge of the substrate to prevent the charge repulsion between the negative charge of the substrate and the oxyanion, which indirectly stabilizes the oxyanion and eventually enhances the catalytic rate (Fig. 8).

In conclusion, based on the kinetic and structural analyses of MAE2, we demonstrated that the positively charged guanido group of Arg-158 is critically required not only to recognize the substrate but also to stabilize the transition-state oxyanion through stabilizing the negative charge adjacent to the oxyanion. Through the formation of stable ion pairs with the negative charges of the substrate, Arg-158 could play dual roles in both the substrate recognition and the acceleration of the catalytic rate. Our studies on the catalytic mechanism of MAE2 suggest that the guanido moiety of arginine should be critically adapted to efficiently anchor the carboxyl moiety in the enzymatic reaction and that neutralizing negative charges of the substrate which are adjacent to a putative oxyanion be critical to the stabilization of the transition-state oxyanion.

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