Characterization of a Novel Ser-cisSer-Lys Catalytic Triad in Comparison with the Classical Ser-Asp-Asp Triad

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Amidase signature family enzymes, which are widespread in nature, contain a newly identified Ser-cisSer-cis-cis-Lys catalytic triad in which the peptide bond between Ser131 and the preceding residue Gly130 is in a cis configuration. In order to characterize the property of the novel triad, we have determined the structures of five mutant malonamidase E2 enzymes that contain a Cys-cisSer-Lys, Ser-cisSer-Ala-Lys, or Ser-cisSer-Ala triad or a substitution of Gly130 with alanine. Cysteine cannot replace the role of Ser131 due to a hyper-reactivity of the residue, which results in the modification of the cysteine to cysteiny1 sulfinic acid, most likely inside the expression host cells. The lysine residue plays a structural as well as a catalytic role, since the substitution of the residue with alanine disrupts the active site structure completely. The two observations are in sharp contrast with the consequences of the corresponding substitutions in the classical Ser-His-Asp triad. Structural data on the mutant containing the Ser-cisSer-Ala-Lys triad convincingly suggest that Ser131 plays an analogous catalytic role as the histidine of the Ser-His-Asp triad. The unusual cis configuration of Ser131 appears essential for the precise contacts of this residue with the other triad residues, as indicated by the near invariance of the preceding glycine residue (Gly130), structural data on the G130A mutant, and by a modeling experiment. The data provide a deep understanding of the role of each residue of the new triad at the atomic level and demonstrate that the new triad is a catalytic device distinctively different from the classical triad or its variants.

A large group of enzymes, designated as amidase signature (AS) family enzymes, are widely distributed throughout the hierarchy of living organisms. A search against the Swiss-Prot data base with the BLAST algorithm (1) identifies over 200 different AS family proteins from 90 different organisms. These enzymes are characterized by the AS sequence, which is a conserved stretch of ~130 amino acids (2, 3). The identified biochemical function of the AS family enzymes has commonly been the hydrolysis of the amide bond (CO–NH2). The known biological functions of the AS family enzymes vary widely, including the formation of Glu-tRNA51Glu via amidolysis of glutamine in many different bacteria (4, 5), catabolism of neuromodulatory fatty acid amides in mammals (6–9), the formation of indole-3-acetic acid in pathogenic plant bacteria (10), and the metabolic turnover of carbon/nitrogen-containing compounds in both prokaryotes and eukaryotes (2, 11). One member of this family, nitrile hydratase, is used in the industrial scale production of acrylamide and nicotinamide (12).

Despite the functional variety and the wide evolutionary distribution of the AS family enzymes, which rival that of the other classical serine hydrolases, e.g. trypsin-like serine protease family containing about 270 different members in the Swiss-Prot data base, little is known about the structure-function relationship of the AS family enzymes. Recently, we reported the structure of malonamidase E2 (MAE2) from Bradyrhizobium japonicum, the first structure of an AS family enzyme (13). The structure revealed a novel catalytic triad Ser-cis-cis-Ser-Lys. The peptide bond between the second serine, Ser131, and the preceding residue, Gly130, is in an unusual cis configuration. The novel catalytic triad had not been observed in any other known hydrolytic enzymes. Subsequently, the structures of two other AS family enzymes have been reported: peptide amidase of Stenotrophomonas maltophilia (11) and rat fatty acid amide hydrolase (15), both of which revealed the cis configuration of Ser131. The triad residues, found exclusively on the AS sequences, are absolutely conserved, and no variation of the chemical makeup of the triad has been observed. This is in sharp contrast with the classical Ser-Asp-Asp triad, which is often found in several completely dissimilar protein folds (16), and whose variations are found in similar protein folds, such as the Cys-His-Glu triad in human rhinovirus 3C protease, an α-chymotrypsin-like protease (17) and Ser-Asp-Glu triad in a fungal lipase (18). While the classical triad has been characterized in minute detail, little is known about the characteristics of the Ser-cis-cis-Ser-Lys triad. In this report, we provide a structural basis for understanding the invariability of the chemical makeup of the novel triad and delineate the role of each constituent amino acid in detail. This study led to the conclusions that the novel triad is a catalytic device distinctively different from the classical triad or its variants, and for this reason, that the AS family is evolutionary distinct from any other known hydrolase families.
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The numbers in parentheses are statistics from the highest resolution shell.

| Parameters                  | S155C (sulfinated) | S131A/ malonate | S131A/ malonate | K62A | G130A | T150A |
|-----------------------------|--------------------|-----------------|-----------------|------|-------|-------|
| Resolution (Å)              | 20.18              | 20.23           | 20.18           | 20.20| 20.22 | 20.19 |
| Rmerge (%)                  | 6.1 (28.3)         | 8.6 (23.1)      | 5.3 (19.8)      | 6.9 (21.7)| 6.6 (25.4)| 7.5 (28.1) |
| Total reflections           | 485345             | 345190          | 514152          | 384083| 712191| 694273 |
| Unique reflections          | 69397              | 33684           | 71433           | 59620 | 38209 | 58083 |
| Completeness (%)            | 93.3 (80.5)        | 90.1 (79.8)     | 94.3 (84.7)     | 89.0 (76.1)| 90.7 (79.3)| 91.7 (81.3) |
| Number of refined atoms     |                    |                 |                 |      |       |       |
| Protein/water               | 6128/393           | 6122/940        | 6122/564        | 6116/947| 6126/938| 6120/946 |
| R-factor/Rmerge (%)         | 0.19/42.7          | 0.19/26.5       | 0.19/22.6       | 0.18/23.7| 0.17/21.2| 0.17/22.9 |
| Rmds bond length (Å)        | 0.0051             | 0.0092          | 0.0048          | 0.0032| 0.0048| 0.0049 |
| Rmds bond angle (°)         | 1.252              | 0.900           | 1.291           | 1.034 | 1.244 | 1.256 |
| Ramachandran plot (%)       |                    |                 |                 |      |       |       |
| Most favored region         | 90.4               | 88.3            | 91.4            | 90.3  | 89.3  | 89.9  |
| Additionally allowed region | 9.0                | 11.3            | 8.4             | 9.7   | 11.0  | 9.9   |
| Generously allowed region   | 0.1                | 0.4             | 0.1             | 0.0   | 0.1   | 0.2   |

*M Rmerge = \( \frac{\sum_{i=1}^{N} |I_{obs} - I_{calc}|}{\sum_{i=1}^{N} I_{obs}} \), where \( I_{obs} \) is the observed intensity of individual reflection and \( I_{calc} \) is average over symmetry equivalents.

** Completeness for \( I/\sigma(I) > 1.0 \).

** R-factor = \( \frac{\sum_{i=1}^{N} |F_{o} - F_{c}|^2}{\sum_{i=1}^{N} |F_{o}|^2} \), where \( F_{o} \) and \( F_{c} \) are the observed and calculated structure factor amplitudes, respectively. Rfree was calculated with 5% of the data.

RESULTS AND DISCUSSION

Overall Structure and Catalytic Mechanism—MAE2 is a dimeric protein. The structures of the two subunits are essentially the same. Previously, we showed that the absolutely conserved Ser131, Ser132, and Lys158 constitute a catalytic triad, and Ser152 is the nucleophile initiating the catalysis (13). The conserved AS sequence forms roughly the core of the molecule and interacts with a substantial part of the remainder of the sequence (Fig. 1A). The two most highly conserved segments on the AS sequence are Gly130-Gly-Ser-Ser-Gly134 and Thr150, Gln-Thr-Gly-Gly-Ser156 (Fig. 1B). The first segment is the loop containing the cis peptide bond between Gly130 and Ser131. The unusual cis configuration allows two direct hydrogen-bonding interactions between Ser131 and Ser156 Oγ (Fig. 1A). The second conserved segment contains the oxanyan hole composed of the backbone -NH groups of Thr152, Gly153, Gly154, and Ser155 (Fig. 1A). Lys62 is in direct contact with the two loops via the interactions with the side chains of Ser131, Ser132 and Thr150.

The catalytic mechanism of the classical Ser-His-Asp triad is known to begin with the polarization of the serine residue by the histidine residue, which is initially in the deprotonated form that is able to abstract the hydroxyl proton of the serine residue (22). The aspartate promotes the base role of the histidine by stabilizing the charged imidazole of the histidine. The same mechanism is not conceivable for the Ser-cisSer-Lys triad because the chemical property of the Ser-Lys pair is very different from that of the His-Asp pair. Since the pK_a of serine is around 13, cisSer131 of the Ser-cisSer-Lys triad is most likely in the protonated form at physiological pH and is unlikely to function as a general base to abstract a proton from Ser156. The chemical environment around Ser131 Oγ is not unusually polar to promote deprotonation of the residue. Rather, C8 of Ala111, which is only 3.4 Å apart from Ser131 Oγ, renders the environment partly hydrophobic. We proposed that Ser155 is in the deprotonated state per se mainly by virtue of the two direct hydrogen-bonding interactions provided by the backbone -NH and the side chain -OH groups of cisSer131 (Fig. 1A) and by the additional dipolar interactions provided by the oxanyan hole and the guanidine group of Arg158 (13). Upon the nucleophilic attack of Ser155 on the carbonyl carbon of the substrate, Ser131 is proposed to function as the catalytic acid that provides a proton to the leaving amino group, which is firmly supported by

MATERIALS AND METHODS

Site-directed Mutagenesis and Protein Purification—S155C, S131A, K62A, G130A, and T150A MAE2 mutants were created using the QuickChange kit (Stratagene). The plasmids containing each of the mutant MAE2 genes was transformed into Escherichia coli strain BL21(DE3). The expression of each of the MAE2 mutants was induced by 1 mM isopropyl-β-D-thiogalactopyranoside at an optical density of 0.6–0.8 at 37 °C for 16 h except for the G130A mutant, which required the induction at 24 °C. Bacterial lysates were prepared by sonication in buffer A, 20 mM Tris-HCl solution (pH 7.4) containing 15% glycerol. After centrifugation, the supernatant was applied to a HiTrap™ Blue HP column (Amersham Biosciences) and eluted with a linear gradient from 0 to 2 M NaCl in buffer A. The fractions containing MAE2 were dialyzed against buffer B, 20 mM Tris-HCl solution (pH 7.4) containing 2 M NaCl. The dialyzed protein sample was loaded onto the RE-SOURCYT™ PHE column (Amersham Biosciences) pre-equilibrated with buffer B containing 2 mM ZnSO4. The unbound fraction containing MAE2 was concentrated and subsequently loaded on a Superdex 200 HR 20/60 column (Amersham Biosciences) pre-equilibrated with 20 mM Tris-HCl (pH 7.4). The peak fractions were concentrated to 10 mg/ml using centricon-10K (Amicon). For the purification of the S155C mutant, we included 1 mM dithiothreitol in all the buffers used.

Crystallization, X-ray Data Collection, and Structure Determination—The crystals of the MAE2 mutants were obtained in droplets containing 1.5 μl of protein sample (10 mg/ml) and an equal volume of precipitant solution containing 20% polyethylene glycol 1000 and 0.1 M Tris-HCl (pH 7.0). The crystals of the S131A MAE2 mutant in complex with malonamate or with malonate were obtained by the cocrystallization with the side chains of Ser131 and Ser155 Oγ, which is only 3.48 Å different from that of the His-Asp pair. Since the pK_a of serine is around 13, cisSer131 of the Ser-cisSer-Lys triad is most likely in the deprotonated form at physiological pH and is unlikely to function as a general base to abstract a proton from Ser156. The chemical environment around Ser131 Oγ is not unusually polar to promote deprotonation of the residue. Rather, C8 of Ala111, which is only 3.4 Å apart from Ser131 Oγ, renders the environment partly hydrophobic. We proposed that Ser155 is in the deprotonated state per se mainly by virtue of the two direct hydrogen-bonding interactions provided by the backbone -NH and the side chain -OH groups of cisSer131 (Fig. 1A) and by the additional dipolar interactions provided by the oxanyan hole and the guanidine group of Arg158 (13). Upon the nucleophilic attack of Ser155 on the carbonyl carbon of the substrate, Ser131 is proposed to function as the catalytic acid that provides a proton to the leaving amino group, which is firmly supported by activity was not detected, the reaction time was extended to 90 min. Any cloudiness was removed by centrifugation prior to the absorbance measurement at 540 nm.
the present work described below. The proton transfer should be facilitated by the amino group of Lys62 that can stabilize the deprotonated form of Ser131. The proposed mechanism is depicted in Fig. 2. Although we will not rule out other possibilities of polarizing Ser155 of the triad, the proposed mechanism may best explain the role of the cis peptide bond; allowing the two groups of Ser131 to simultaneously polarize Ser155.

Cysteine Cannot Replace the Role of Ser155—In cysteiny1proteases, the serine nucleophile is substituted with cysteine. Several hydrolases of this family, including human ubiquitin C-terminal hydrolase (23), tobacco etch virus protease (24), and turnip mosaic potyvirus NIa (25), are known to contain a Cys-His-Asp triad. Many papain-like cysteine protease family members utilize a Cys-His-Asn catalytic triad. The asparagine residue of the triad was shown to be nonessential for catalysis at least in papain (26), which is probably a reflection of the higher reactivity of cysteine than serine. We tested the suitability of sulfur to function as a nucleophile in the active site environment of MAE2 by mutating Ser155 to cysteine. Although the cysteine thiol is chemically more reactive than the serine hydroxyl in general, the S155C MAE2 mutant was inactive. The structure of the mutant enzyme provides a clear explanation to this observation. Cys155 had extra density extending from the sulfur atom in the initial electron density map at 1.8 Å resolution (Fig. 3). The chemical environment in the vicinity of the extra density immediately indicated that the cysteine residue was modified as cysteine sulfinic acid, Cys-SO2H. The side chain of this residue directly interacts with the oxyanion hole and the backbone -NH of Ser131. Cysteine sulfinic acid is an oxidation product of cysteine sulfenic acid, Cys-SOH, which is produced from the reaction of a cysteine residue with a peroxide molecule (27). While cysteine sulfenic acid is generally unstable and could be reduced to back to cysteine in the presence of dithiothreitol, cysteine sulfinic acid is not reactive to the reducing agent (28, 29). Consistently, the enzyme activity of this mutant was not restored in the presence of 10 mM dithiothreitol. Although we included 1 mM dithiothreitol in all buffer solutions used for the purification of the mutant enzyme, the bacterial cell lysate as well as the purified mutant enzyme were enzymatically inactive. Therefore, Cys155 must have been modified to Cys-SOH inside the cells used for the production of the mutant protein, and the further oxidation to Cys-SO2H might have also taken place during the expression of the protein. Since the active site is composed of the most highly conserved peptide segments, both the loop containing cisSer131 and the

FIG. 1. Structure and sequence alignment. a, structure of MAE2. The conserved AS sequence is highlighted in red and the nonconserved region is in blue. The three triad residues, which are on the AS sequence, are in yellow ball-and-sticks. The inset shows the close-up view of the region of the catalytic triad. Hydrogen bonds are depicted as dashed lines. b, sequence alignment of six AS family members from low to high organisms. The alignments are shown for MAE2, a bacterial Glu-tRNA\(^{\text{AAA}}\) amidotransferase (GluAT, gi 2589195), a putative yeast amidase (Amd2p, gi 6320448), a putative Caenorhabditis elegans amidase (Cegel, gi 6425411), chicken vitamin D\(_3\) hydroxylase-associated protein (VDHAP, gi 1079452), and human fatty acid amide hydrolase (FAAH, gi 4557575). The three triad residues are in red. The color bars indicate the sequence conservation in 20 selected AS family enzymes that include the above six enzymes and others whose biochemical functions are different or unknown.

FIG. 2. Proposed catalytic mechanism of the MAE2. The catalysis proceeds through the formation and subsequent breakdown of the covalent acyl-enzyme intermediate. The oxyanion hole composed of the four backbone -NH groups stabilizes the tetrahedral transition states. Ser155 is proposed to be in the deprotonated state per se at neutral pH.
segment containing the oxyanion hole should adopt very similar conformations in all AS family members. Presumably, cysteine has not been selected as the catalysis-initiating nucleophile by any AS family enzymes because the active site milieu renders the cysteine residue at this position highly reactive, which is unwanted and uncorrectable.

The substitution of the serine of the Ser-His-Asp triad with cysteine results in a 10^6-fold decrease in the catalytic activity of trypsin. A crystallographic study showed that the cysteine is in the reduced state, and the bulkiness of the cysteinyll sulfur atom obstructs the oxyanion hole (30). This obstruction was suggested to be the main structural ground for the reduced activity of the mutant. The oxyanion hole of MAE2 consists of four backbone-NH groups (Fig. 3), which appears to readily accommodate an oxygen atom of a peroxide molecule to provide a low energy barrier for the reaction of Cys155 or Cys155-OH with the other oxygen atom of the molecule. Two subsequent reactions of Cys155 with peroxide molecules can lead to the formation of Cys155-SO_2H. In contrast, the oxyanion hole of trypsin consists of two backbone -NH groups and apparently does not support the oxidation of the substituted cysteine to cysteine sulfenic acid or the further oxidation.

The Catalytic Roles of Ser^{131}—The structures of the S131A mutant were determined as a complex with the substrate malonamate (OOC–CH_2–CO–NH_2) and with the product malonate (OOC–CH_2–COO^-). The shape of the electron density of the bound substrate is readily distinguishable from that of the bound product (Fig. 4). Therefore, the hydrolysis of the substrate by the mutant enzyme did not take place during several days of the substrate soaking into the crystals of the mutant enzyme. The mutant exhibits about 2000-fold lower catalytic activity. The mutant exhibits about 2000-fold lower catalytic activity. The crystallization conditions might have reduced the activity significantly further.

Role of the Conserved Glycine Preceding Ser^{131}—Gly^{130} is strictly conserved in all but two AS family proteins, which contain the substitution of the glycine residue with alanine (access code: gi13507185) or valine (access code: gi15610511), respectively. Since the peptide bond between Gly^{130} and Ser^{131} is in the cis configuration, we investigated whether substitution of Gly^{130} with alanine may result in the trans peptide bond between the two residues with a concurrent defect in the catalytic activity. The structure of the G130A mutant was determined at 2.2 Å. From the beginning of the refinement of the structure, the electron density for Ala^{130} was barely visible and that for Gly^{129} and Ser^{131} was fairly weak, indicating that the substitution results in the unstable conformation of this segment, in contrast with the well-defined conformation of this segment in the wild-type enzyme. Although weak or fragmental, the electron density could be explained by a conformation of the peptide bond between Ala^{130} and Ser^{131} in the cis configuration (Fig. 5). This is true for both MAE2 molecules in the asymmetric unit of the crystal. Alani ne modeled at the position of residue 130 in the structure of the wild-type enzyme shows that its Cβ atom is too close to the Ca atom of cisSer^{131} (2.9 Å, data not shown). This indicates that Ala^{130} has to adopt backbone torsion angles different from those of Gly^{130} to avoid steric clash. Apparently, the Ala^{130}-containing loop cannot form a rigid structure by adopting an alternative conformation. The “shaking” loop conformation in the mutant enzyme results in 6-fold decrease in k_cat and 2-fold increase in the Michaelis constant K_m. Most likely, the ability of the mutant to adopt the cis configuration avoids a chaotic failure in the enzyme activity. The data explain why Gly^{130} is virtually invariable and also why the two exceptions to the conservation of the glycine residue are allowed. When the trans peptide bond was modeled between Gly^{130} and Ser^{131}, the -NH group of Ser^{131} cannot form a hydrogen bond with Ser^{135} Oγ and the -OH group of Ser^{131} cannot make the precise and simultaneous contacts with Ser^{135} and Lys^{58} that are observed for the cis configuration. All these
observations suggest that the cis configuration of Ser\textsubscript{131} is essential for the catalytic function of the triad.

Lys\textsubscript{62} Plays Catalytic and Structural Roles—The direct interaction of the amino group of Lys\textsubscript{62} and the hydroxyl group of Ser\textsubscript{131} suggests that Lys\textsubscript{62} plays the catalytic role of stabilizing the Ser\textsubscript{131} alkoxide in the course of the catalysis. The structure of the K62A mutant reveals that the lysine residue of the triad plays a critical structural role as well as the proposed catalytic role. In the structure of the wild-type enzyme, the amino group of Lys\textsubscript{62} is in direct contact with Ser\textsubscript{132} and Thr\textsubscript{150} (Fig. 1a), which are conserved as either threonine or serine. Thr\textsubscript{150} O\textsubscript{Y} interacts indirectly with Thr\textsubscript{152} O\textsubscript{Y} via a bound water molecule (Wat\textsubscript{215}), forming a hydrogen-bonded network of Lys\textsubscript{62}. Thr\textsubscript{150}. Wat\textsubscript{215}. Thr\textsubscript{152}. The substitution of the serine residue with alanine in fatty acid amide hydrolase corresponding to Ser\textsubscript{132} decreased the catalytic activity by \(100\)-fold (31), while the substitution of the threonine residue with alanine in the peptide amidase corresponding to Thr\textsubscript{150} decreased the catalytic activity by \(3\)-fold (14). The absence of these two interactions in the K62A mutant prompts a significant rearrangement of the segment containing the oxyanion hole, which forms several new interactions with surrounding residues and water molecules, as deduced from the unambiguous electron density of the active site (Fig. 6a). Notably, Ser\textsubscript{155} is completely out of place from its correct position and interacts with the -NH group of Ile\textsubscript{157}. Furthermore, the loop comprising the oxyanion hole is also distorted compared with that in the wild-type enzyme. It is hard to imagine that the binding of the substrate could trigger a restoration of the catalytically competent active site structure. The complete spoiling of the active site clearly explains why the K62A mutant exhibits no catalytic activity. In order to probe the importance of the hydrogen bond between Lys\textsubscript{62} and Thr\textsubscript{150} in maintaining the structure of the segment containing the oxyanion hole, which forms several new interactions with surrounding residues and water molecules, as deduced from the unambiguous electron density of the active site (Fig. 6a). Notably, Ser\textsubscript{155} is completely out of place from its correct position and interacts with the -NH group of Ile\textsubscript{157}. Furthermore, the loop comprising the oxyanion hole is also distorted compared with that in the wild-type enzyme. It is hard to imagine that the binding of the substrate could trigger a restoration of the catalytically competent active site structure. The complete spoiling of the active site clearly explains why the K62A mutant exhibits no catalytic activity. In order to probe the importance of the hydrogen bond between Lys\textsubscript{62} and Thr\textsubscript{150} in maintaining the structure of the segment containing the oxyanion hole, we determined the structure of the T150A mutant enzyme. The structure reveals that two water molecules (Wat\textsubscript{215} and Wat\textsubscript{386}) relocate to fill in the space vacated by the mutation and maintain the indirect interaction between Lys\textsubscript{62} and Thr\textsubscript{152} (data not shown). The loop structure of the oxyanion-containing segment of this mutant is nearly the same
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as that of the wild-type enzyme. Consistently, the mutant enzyme exhibits about one-fourth of the catalytic activity of the wild-type enzyme (Fig. 7), which is similar to the consequence of the corresponding mutation in the peptide amidase (14). We speculate that the hydrogen-bonded network that links Lys$^{62}$ and the oxyanion-containing segment via Thr$^{150}$ is important for maintaining the active site structure, but the importance is masked by the salvaging water-mediated hydrogen bonds in this mutant. In the K62A mutant, the link is lost by the absence of the long side chain of Lys$^{62}$, which allows the direct interaction between Ser$^{132}$ and Thr$^{150}$, and the penetration of two water molecules into the vacated site (Fig. 6a). Two tightly bound water molecules, Wat$^{242}$ and Wat$^{244}$, indicated as Wat1 and Wat2, are absent in the wild-type MAE2 structure. The hydrogen bonds present only in the mutant structure are shown in dotted lines. a, superposition of the active sites. The catalytic components of the wild-type (in coral) and K62A mutant (in blue) enzymes are superimposed. The circles indicate the positions of the Ser$^{155}$ in the two structures. Unlike the oxyanion hole-containing segment, the cisSer$^{131}$-containing loop undergoes a minor conformational change. Figs. 1–6 were prepared using the program BobScript and rendered using Raster3D.

Fig. 6. K62A mutation disrupts the active site. a, 2F$_{o}$ – F$_{e}$ electron density map for the active site of the K62A mutant. The map was calculated to 2.0 Å and contoured at 1.5 σ. Two tightly bound water molecules, Wat$^{242}$ and Wat$^{244}$, indicated as Wat1 and Wat2, are absent in the wild-type MAE2 structure. The hydrogen bonds present only in the mutant structure are shown in dotted lines. b, superposition of the active sites. The catalytic components of the wild-type (in coral) and K62A mutant (in blue) enzymes are superimposed. The circles indicate the positions of the Ser$^{155}$ in the two structures. Unlike the oxyanion hole-containing segment, the cisSer$^{131}$-containing loop undergoes a minor conformational change. Figs. 1–6 were prepared using the program BobScript and rendered using Raster3D.

In contrast with the consequence of the K62A mutation, the substitution of the aspartic acid with alanine of the Ser-His-Asp triad does not result in a complete loss of the enzyme activity of subtilisin (32). The mutation affects the general acid/base catalytic function, but it does not affect the integrity of the active site structure, as the mutant exhibited only a small increase in $k_{m}$ (−2-fold). Cytomegalovirus protease contains a Ser-His-triad, and the stereocchemical positions of the catalytic components of the enzyme are essentially the same as those of trypsin-like proteases (33). These observations indicate that the aspartic acid in the Ser-His-Asp triad plays the catalytic role only, while the lysine residue in the Ser-cisSer-Lys triad plays the structural role as well as the catalytic role. Probably, the dual function, which the third residue of the novel triad serves, has suppressed selection of other amino acids by the AS family enzymes during evolution.

Fig. 7. Effect of site-directed mutagenesis on the catalytic efficiency. The $k_{cat}$ values of the wild-type MAE2 and mutant enzymes are shown in relative scale. The measured $k_{cat}$ value of the wild-type enzyme is 1844 ± 178 s$^{-1}$ and that of the S131A mutant is 1.07 ± 0.04 s$^{-1}$. The activities of the S155C and K62A mutants were undetectable.

CONCLUSIONS

The study presented here delineates the role of each residue of the Ser-cisSer-Lys triad in detail and addresses the question of why the chemical make-up of the triad is absolutely conserved. We showed that the Cys-cisSer-Lys triad is not functional because the cysteine residue is modified to cysteine sulfinic acid. The active site environment of MAE2 appears to readily promote the oxidation of Cys$^{155}$ to cysteine sulfinic acid. The structures of the S131A mutant in complex with the product and with the substrate demonstrate that Ser$^{131}$ is the catalytic acid protonating the leaving amino group of the substrate. The consequent ion-pair formation between the serine alkoxide and the amino group of the lysine would facilitate the proton transfer reaction. The cis configuration of Ser$^{131}$ appears essential for the formation of the two direct hydrogen bonds with Ser$^{155}$ and another with Lys$^{62}$. A simple modeling experiment suggests that any residue bulkier than serine at this position would disrupt at least the structure of the active site. The third lysine residue was shown to play a critical structural role of propping the active site structure as well as the proposed catalytic role of promoting the protonation of the leaving group by Ser$^{131}$ of the triad. The dual role appears to be supported only by lysine at this position that has to make precise contacts with the three different conserved residues. Currently, the structures of three AS family enzymes are available. The AS sequences of the three enzymes comprise the catalytic core scaffolds that are very similar to each other. The orientations of the catalytic components, the triad, and the oxyanion hole-containing segment of the three enzymes are superimposable with root mean square deviations less than 0.32 Å. We, therefore, suggest that the conclusions drawn from this mutational-structural study would be generally true for the whole AS family enzymes.

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Characterization of a Novel Ser-cisSer-Lys Catalytic Triad in Comparison with the Classical Ser-His-Asp Triad
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