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# Mutational Analysis of Residues in Two Consensus Motifs in the Active Sites of Cathepsin E[1]

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**Cathepsin E, an intracellular aspartic proteinase of the pepsin family, is composed of two homologous domains, each containing the catalytic Asp residue in a consensus DTG motif. Here we examine the significance of residues in the motifs of rat cathepsin E by substitution of Asp98, Asp283, and Thr284 with other residues using site-directed mutagenesis. Each of the mutant proenzymes, as well as the wild-type protein, was found in culture media and cell extracts when heterologously expressed in human embryonic kidney 293T cells. The single mutants D98A, D283A, and D283E, and the double mutants D98A/D283A and D98E/D283E showed neither autocatalytic processing nor enzymatic activities under acidic conditions. However, the D98E and T284S mutants retained the ability to transform into the mature forms, although they exhibited only about 13 and 40% of the activity of the wild-type enzyme, respectively. The $K_m$ values of these two mutants were similar to those of the wild-type enzyme, but their $k_{cat}$ values were greatly decreased. The $K_i$ values for pepstatin and the *Ascaris* pepsin inhibitor of the mutants and the wild-type enzyme were almost the same. The circular dichroism spectra of the two mutants were essentially the same as those of the wild-type enzyme at various pH values. These results indicate that (i) Asp98, Asp283, and Thr284 are indeed critical for catalysis, and (ii) the decrease in the catalytic activity of D98E and T284S mutants is brought about by an effect on the kinetic process from the enzyme-substrate complex to the release of the product.**

**Key words: active-site motif, aspartic proteinase, cathepsin E, characterization of mutants, correct folding.**

Animal members of the pepsin family include secretory enzymes such as pepsin and renin and nonsecretory enzymes such as cathepsin D and cathepsin E. They are known to be composed of two homologous domains, despite having very little amino acid sequence similarity (reviewed in Refs. *1–3*). Each domain contains one of the pair of catalytic Asp residues which reside in a consensus D(T/S)G motif. Cathepsin E is a major intracellular aspartic protease that exists as a homodimer of two catalytically identical monomers with a molecular mass of 42 kDa (*4–8*). Among the pepsin family of aspartic proteinases, cathepsin E is unique in its limited tissue distribution in certain cell types such as lymphoid tissues, gastrointestinal tracts, urinary organs, and blood cells (*9, 10*) and its cell-specific localization (*11–14*). Cathepsin E is also unique in its structure. The amino terminal portion contains a Cys residue at position 43, which responsible for a disulfide bond formation between the two identical subunits. The homodimeric form is easily converted into the monomeric form exhibiting the full catalytic activity under reducing conditions (*5, 15, 16*).

The highly conserved tripeptide sequence DTG in two active sites is found in cathepsin E from all of species (*8, 15, 17*), except that the tripeptide sequence near the N-terminal region of rabbit cathepsin E is replaced by DTV (*18*). The catalytic activity of cathepsin E is inhibited strongly by pepstatin, a common inhibitor of aspartic proteinases, and specifically by *Ascaris* pepsin inhibitor. Recently, a novel transmembrane aspartic proteinase, BACE-1 (beta-site APP cleaving enzyme), has been identified (*19–22*). This enzyme also contains two active site motifs [D(T/S)G]. However, in addition to its structural characteristics, BACE-1 is unique among members of the aspartic proteinase family with respect to its substrate preference and inhibitor profiles (*23*). Namely, BACE-1 has a loose substrate specificity and is not sensitive to pepstatin.

Although two aspartic acids have been identified as the catalytic residues of cathepsin E, the role in the correct folding and the catalytic activity of other amino acid residues in the two active-site motifs is currently unknown. Because the tertiary structure of cathepsin E is also unknown, analysis of the conserved active-site residues is the most practical approach for understanding the catalytic mechanism of cathepsin E. In this study, seven mutants of

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Abbreviations: CD, circular dichroism; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; Dnp, *N*-2,4-dinitrophenyl; MOCAc, (7-methoxycoumarin-4-yl)acetyl; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

cathepsin E were constructed by replacing one or two residues of the two active-site motifs using site-directed mutagenesis, and the mutant proteins were heterologously expressed in human embryonic kidney (HEK) 293 cells. The enzymatic activity and the capability of folding and autoprocessing of the mutant proteins were analyzed.

## MATERIALS AND METHODS

*Materials*—Antibodies specific for rat cathepsin E were raised in rabbits and purified by affinity chromatography as described previously (*24*). Polyclonal antibodies against the synthetic peptide Ser-Gln-Leu-Ser-Glu-Phe-Trp-Lys-Ser-His-Asn-Leu-Asp-Met, which corresponds to the prosequence comprising residues $Ser^{23}$-$Met^{36}$ of human procathepsin E, were raised in rabbits and purified on the peptide-Sepharose affinity column as described previously (*25*). The purified antibodies cross-reacted with procathepsin E only. The fluorogenic synthetic substrate MOCAc-Gly-Lys-Pro-Ile-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-$NH_2$ was synthesized as described (*26*). The pcDNA 3.0 was purchased from Invitrogen.

*Plasmid and Mutant Constructions*—The pBluescript II $SK^-$ plasmid containing full-length rat cathepsin E cDNA between the *Sma*I and *Xho*I sites was described previously (*8*). The active-site residues were changed by site-directed mutagenesis according to the method of Kunkel *et al.* (*27*) with some modifications. The oligonucleotide primers used in the mutagenesis reaction were as follows: primer 1 (for construction of D98A), 5′-ATCTTTGCCACGGGCTCATC-3′, primer 2 (for construction of D283A), 5′-ATAGTGGCAACAGGGACC-3′, primer 3 (for construction of T284S), 5′-CCATAGTGGACTCAGGGACCTCC-3′, primer 4 (for construction of D98E), 5′-GTCATCTTTGAGACGGGCTCATC-3′, primer 5 (for construction of D283E), 5′-GCCATAGTGGAGACAGGGACCTC-3′. The D98A/D283A mutant was produced by cleaving plasmids containing D98A and D283A with *Mun*I and *Xho*1 restriction enzymes and ligating the two fragments containing D98A and D283A, respectively. The D98E/D283E mutant was produced in the same way as described above. All constructs were verified by DNA sequence analysis. The wild-type and mutant cDNAs were subligated into pcDNA 3.0 plasmid for heterologous expression in mammalian cells.

*Expression of Cathepsin E Proteins in HEK293 Cells*—HEK 293T cells, which contained little or no cathepsin E under normal conditions, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 mg/ml streptomycin (complete DMEM) at 37°C in a 5% $CO_2$ incubator. The expression plasmid DNA (10 mg per 100-mm tissue culture plate) was transfected into HEK 293T cells by the calcium phosphate precipitation method (*28*). The cells were grown to approximately 90% confluence by overnight incubation in complete DMEM. The medium was then replaced with serum-free Opti-MEM (5 ml/plate) and incubation was continued for an additional 48 h. The expression of wild-type and mutant proteins in cells was confirmed by immunoblotting of cell extracts. Under these conditions, 20–30% of the total cathepsin E activity in each culture was recovered in the culture supernatant.

*Protein Purification*—After centrifugation at 16,000 ×*g* for 20 min, the culture media were collected. The cells were washed twice with phosphate-buffered saline (PBS), removed from the plates with a rubber scraper, and subjected to centrifugation at 300 ×*g* for 5 min. The sedimented cells were suspended in PBS containing 0.1% Triton X-100, sonicated for 1 min at 4°C, and subjected to centrifugation at 100,000 ×*g* for 1 h. The resultant supernatant fraction is referred to as the cell lysate.

The wild-type cathepsin E and the T284S and D98E mutant proteins were purified from the respective culture media by a slight modification of the scheme described for rat spleen (*4*). Briefly, the culture media were concentrated and adjusted to pH 3.5 with 0.1 M sodium acetate buffer containing 0.05% Brij 35 and 1 M NaCl. After concentration, the supernatants were applied to a column of pepstatin A–Sepharose 4B equilibrated with 0.1 M sodium acetate buffer at 3.5, containing 0.05% Brij 35 and 1 M NaCl. The column was washed with the same buffer, then the adsorbed protein was eluted with 0.1 M Tris-HCl buffer at pH 8.6, containing 0.05% Brij35 and 1 M NaCl. The adsorbed fraction was concentrated and dialyzed against 20 mM sodium phosphate buffer, pH 7.0, containing 0.1% Brij 35. The enzyme solution was applied to a Mono Q column equilibrated with 20 mM sodium phosphate buffer, pH 7.0, containing 0.05% Brij 35 and eluted with a gradient of NaCl ranging from 0 to 1 M.

*Enzyme Assays and Protein Determination*—Acid protease activity was measured at pH 3.5 using 1.5% acid-denatured hemoglobin as a protein substrate as described previously (*6*). In some experiments, the fluorogenic synthetic substrate MOCAc-Gly-Lys-Pro-Ile-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-$NH_2$ was used for the assay of cathepsin E according to the method as described previously (*26*). Kinetic parameters for the hydrolysis of the fluorogenic substrate and inhibitor constants for the interaction of cathepsin E with pepstatin A and *Ascaris* pepsin inhibitor (a gift from Dr. Takashi Kageyama, Kyoto University) were determined as described previously (*26, 29*). For the determination of $K_m$ and $V_{max}$ with this synthetic substrate, the same assay was used with variable substrate concentrations. Inhibition constants were determined using this substrate at its $K_m$ and variable concentrations of inhibitors.

*Electrophoresis and Immunoblotting*—Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was performed under reducing conditions in 10% gels after the heat treatment in the solubilizing buffer at 100°C for 5 min as described previously (*29*). For immunoblotting, proteins electrophoresed on SDS gels were transferred electrophoretically to nitrocellulose membranes as described previously (*30*). The blotted membranes were immunostained as described previously (*24*).

*Circular Dichroism Spectra*—The CD spectra were determined at a protein concentration of 0.1 mg/ml in buffer with a Jasco J-720 spectropolarimeter (Japan Spectroscopic, Tokyo) at room temperature using a water-jacketed quartz cell with a light path of 0.1 cm. The sample solutions were prepared by approximately diluting the solutions of the activated enzymes with 20 mM sodium phosphate buffer at pH 7.0 and 0.1 M sodium acetate buffer at pH 5.5 and 4.0, respectively. Ten scans were repeated from 190 to 250 nm under continuous nitrogen flush. The buffer blank and enzyme solutions were degassed prior to the analyses. Secondary structures were determined using the Jasco Protein Secondary Structure Estimation Program

based on the method of Chang *et al.* (*31*).

*Statistical Analysis*—Data are expressed as means ± SD. The significance of differences between groups was determined with two-way analysis of variance (ANOVA), followed by Scheffe's post hoc test for multiple comparison when $F$ ratios reached significance.

## RESULTS

*Production and Characterization of Recombinant Proteins*—To determine the importance of each residue in the active-site motifs of cathepsin E for the autoactivation, catalytic activity, and correct folding, a series of the cathepsin E mutants were constructed by site-directed mutagenesis (Fig. 1) and heterologously expressed in HEK 293T cells. To confirm the catalytic residues, the Asp-98 and Asp-283 residues were substituted with Ala (D98A, D283A, and D98A/D283A) and with another acidic amino acid residue, Glu (D98E, D283E, and D98E/D283E). Further, the Thr-284 residue in the active site motif of the C-terminal domain was substituted with Ser (T284S), since the pepstatin-insensitive BACE 1 contains the DTG and DSG motifs in the N- and C-terminal domains, respectively.

Each of the recombinant proteins expressed in HEK 293T cells was found in both culture media and cells. Immunoblot analysis with antibodies against the mature cathepsin E revealed that each of the mutant proteins in the culture media exhibited the same molecular mass (46 kDa under reducing conditions) as the wild-type enzyme (Fig. 2). All of the proteins also reacted with antibodies against the propeptide of cathepsin E (not shown), indicating that they were proenzymes. The levels of all the mutants in the media were comparable to or somewhat lower than that of the wild-type enzyme. All of the secreted proteins were resistant to endoglycosidase H, indicating that they had complex-type oligosaccharide chains (data not shown).

Procathepsin E is readily autoactivated upon brief incubation at an acidic pH and transforms to the mature form (*15, 32*). To determine whether each of these mutants was capable of transforming to the mature form, the culture media were incubated at 37°C for 10 min in 0.1 M sodium acetate buffer, pH 3.5, at the same protein concentrations.

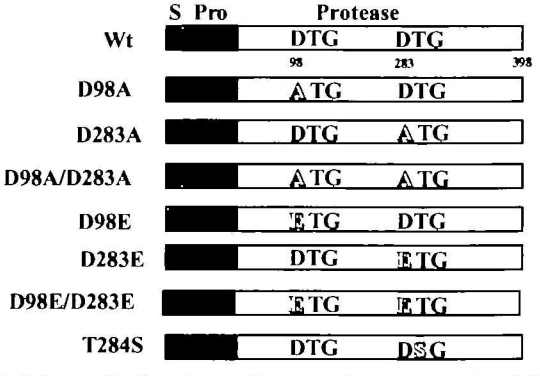


Fig. 1. **Schematic drawings of expression constructs of the active-site mutants of cathepsin E.** Amino acid residues in the two active-site motifs of the wild-type cathepsin E were substituted as shown schematically. The numbers below the wild-type cathepsin E indicate the positions of active-site Asp residues. S, signal sequence; Pro, prosequence; Protease, catalytic domain.

As shown in Fig. 3, the wild-type proenzyme was completely converted into the mature form with an apparent molecular mass of 42 kDa (under reducing conditions). The T284S and D98E mutants were also converted to the 42-kDa forms. Since this conversion was completely inhibited by pepstatin A (not shown), it was considered that these two mutant proteins and the wild-type protein were autoproteolytically processed to generate the respective mature forms. In contrast, D98A, D283A, D98A/D283A, and D98E/D283E showed the resistance to this conversion under the same conditions.

Each of the culture media was analyzed for its ability to hydrolyze acid-denatured hemoglobin as a protein substrate and MOCAc-Gly-Lys-Pro-Ile-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-$NH_2$ as a synthetic substrate. The culture media from the cells expressing either T284S or D98E mutant protein revealed clear enzymatic activities against both substrates, although these were only about 13 and 40% of the activity of the wild-type enzyme, respectively (Fig. 4). The D98A/D283A mutant was not converted into the mature form, even when increasing amounts of the pro-

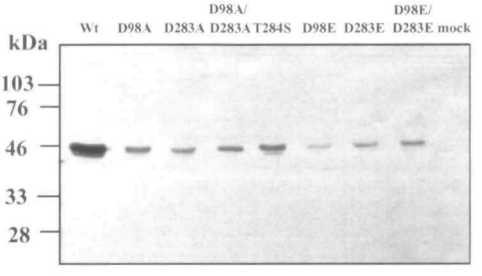


Fig. 2. **Immunoblots from SDS–polyacrylamide gel electrophoresis of the wild-type cathepsin E and the mutant proteins secreted from HEK 293T cells transfected with each construct.** The culture media were collected by centrifugation of HEK 293 T cells expressing the wild-type cathepsin E and the mutant proteins after 48 h of culture. Each culture medium was subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions followed by immunoblotting using polyclonal antibodies against rat mature cathepsin E.

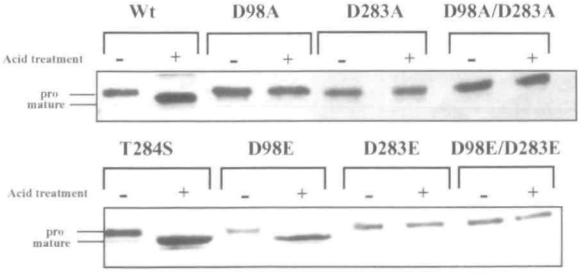


Fig. 3. **Autocatalytic activation of zymogens of the wild-type and mutant enzymes at pH 3.5 analyzed by SDS–polyacrylamide gel electrophoresis under reducing conditions and immunoblotting.** The culture media from HEK 293T cells expressing the wild-type cathepsin E and the mutant proteins were incubated at 37°C for 10 min in 0.1 M sodium acetate buffer, pH 3.5, and then neutralized by addition of 0.1 M Tris-HCl buffer, pH 9.0. After heating at 100°C for 5 min in 1% SDS, the samples were analyzed by SDS–polyacrylamide gel electrophoresis under reducing conditions and immunoblotting using polyclonal antibodies against rat mature cathepsin E.

tein were applied. However, this mutant protein was readily converted to the 42-kDa form and lost its immunoreactivity to antibodies specific for procathepsin E when incubated with a small amount of the wild-type proenzyme at pH 3.5 and 37°C for 10 min (Fig. 5), but it thereby exhibited only the catalytic activity corresponding to the wild-type enzyme added. Similar results were obtained with the D98A, D283A, D283E, and D98E/D283E mutants (not shown). These results indicate that the D98A, D283A, D283E, D98A/D283A, and D98E/D283E mutants are correctly folded in such a way that they do not exhibit the catalytic activity but are cleaved by the mature cathepsin E at the autoproteolytic cleavage site for the N-terminal propeptide.

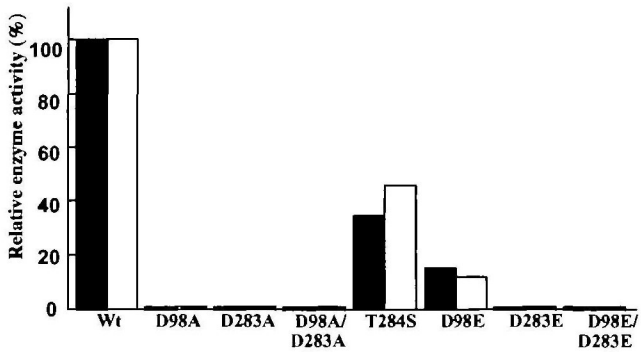


Fig. 4. **Capability of the wild-type and mutant proteins to hydrolyze protein and synthetic substrates.** The culture media from HEK 293T cells expressing the wild-type cathepsin E and the mutant proteins were incubated at pH 3.5 with acid-denatured hemoglobin (closed) at 40°C for 40 min or MOCAC-Gly-Lys-Pro-Ile-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-$NH_2$ (open) at 37°C for 10 min, and the reaction was terminated by addition of 5% trichloroacetic acid. The values are expressed as percentages of the enzyme activities (units/mg protein) of the wild-type enzyme toward each substrate.

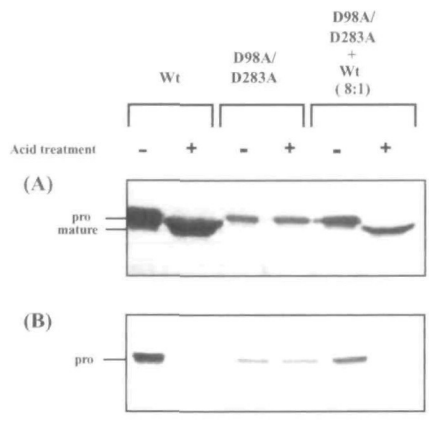


Fig. 5. **Conversion of the zymogen of D98A/D283A double mutant to the mature form by incubation with the wild-type zymogen at pH 3.5.** The culture medium from HEK 293T cells expressing the D98A/D283A mutant was incubated at 37°C for 10 min in 0.1 M sodium acetate buffer, pH 3.5, with and without that expressing the wild-type enzyme at a protein ratio of 8:1. After incubation, the samples were analyzed by SDS–polyacrylamide gel electrophoresis under reducing conditions and immunoblotting with antibodies to the mature domain of cathepsin E (A) and its propeptide (B).

*Purification and Properties of Catalytically acTive Proteins*—The catalytically active mutants T284S and D98E were purified from the respective culture medium by the same method as the wild-type enzyme. For affinity purification on a pepstatin A–Sepharose column, the two mutants and the wild-type protein were subjected to acid treatment at pH 3.5. By this treatment, each protein was autoproteolytically activated and purified as the mature enzyme. The T284S and D98E mutants had about 2.5- and 10-fold lower specific activities, respectively, than the wild-type enzyme (Fig. 4). However, the optimum pH was not influenced by these mutations (not shown). To assess the origin of the suppressed enzymatic activities, the steady-state kinetic parameters, $K_m$ and $k_{cat}$, were determined at pH 4.0 and 37°C using the synthetic substrate. As shown in Table I, the $K_m$ values are almost the same for the two mutants and the wild-type cathepsin E, indicating that the affinity of the substrate is not substantially affected by these mutations. On the other hand, the $k_{cat}$ values were significantly decreased by these mutations, resulting in a large decrease in the $k_{cat}/K_m$ value (2.7-fold for T284S and 8.0-fold for D98E).

Pepstatin A is known to bind efficiently to the active site of most aspartic proteinases but not BACE, whereas *Ascaris* pepsin inhibitor specifically inhibits cathepsin E and pepsin but not cathepsin D. These two inhibitors were used to analyze further the properties of the cathepsin E mutants. As shown in Table II, there was little or no difference in the $K_i$ values for both pepstatin A and *Ascaris* pepsin inhibitor between the wild-type cathepsin E and the two mutants. These results were consistent with the kinetic constants obtained by Lineweaver-Burk plots. Namely, Asp98 and Thr284 can be partially replaced by Glu and

TABLE I. **Kinetic parameters for hydrolysis of the synthetic substrate by cathepsin E and its mutants.** The synthetic peptide MOCAc-Gly-Lys-Pro-Ile-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-$NH_2$ was used as a substrate. Reaction mixtures were incubated at 40°C for 10 min, then the reaction was terminated by addition of 2 ml of 5% trichloroacetic acid. The increase in fluorescence intensity produced by substrate cleavage during incubation was measured at an emission wavelength of 393 nm with excitation at 328 nm using a fluorescence spectrophotometer.

| Enzyme | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($mM^{-1}$ $s^{-1}$) |
|---|---|---|---|
| Wild type | 1.27 | 11.90 | 9.41 |
| T284S | 1.91 | 6.40 | 3.52 |
| D98E | 1.31 | 1.55 | 1.18 |

TABLE II. **Inhibition constants ($K_i$) for the inhibition of wild-type cathepsin E and mutant enzymes by pepstatin A and *Ascaris* pepsin inhibitor.** The substrate was MOCAc-Gly-Lys-Pro-Ile-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-$NH_2$. Enzyme samples were initially mixed with an inhibitor, then incubated with the substrate at 40°C for 10 min. The reaction was terminated by addition of 2 ml of 5% trichloroacetic acid. The $K_i$ values were calculated from experiments with six different concentrations of the substrate. Each inhibitor was competitive in nature and the estimated precision of the values was in the range ± 5–10%.

| Enzyme | $K_i$ (nM) | |
|---|---|---|
| | Pepstatin A | *Ascaris* pepsin inhibitor |
| Wild type | 5.82 | 18.34 |
| T284S | 6.48 | 19.53 |
| D98E | 6.06 | 19.13 |

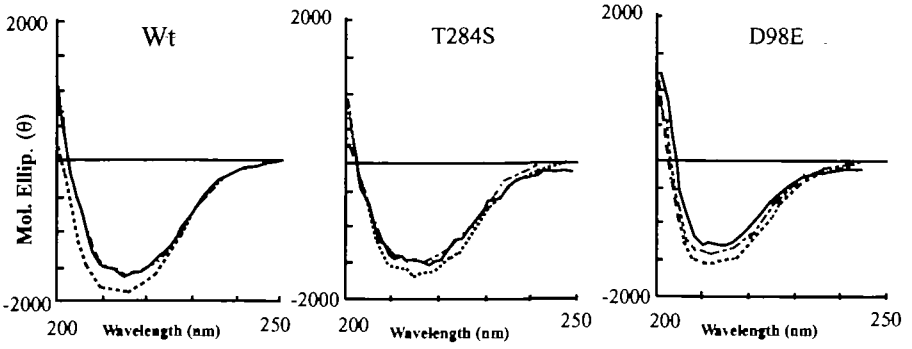


Fig. 6. **CD spectra of the wild-type and mutant proteins.** Spectra were generated using the solution containing the purified wild-type and D98E and T284S mutant proteins (0.1 mg/ml each) at pH 4.0 (—), 5.5 (– – –) and 7.0 (- - -). The buffers used were 0.1 M sodium acetate buffer (pH 4.0 and 5.5) and 20 mM sodium phosphate buffer (pH 7.0). Each sample in a 0.1-cm pathlength cuvette was scanned ten times from 200 to 250 nm at room temperature with continuous nitrogen flush.

Ser, respectively.

To confirm the correct folding of T284S and D98E mutants, the CD spectra of wild-type cathepsin E and the mutants were measured at pH 7.0, 5.5 and 4.0 (Fig. 6). At pH 7.0, the CD spectra of the mutants were indistinguishable from that of the wild-type cathepsin E. At pH 5.5 and 4.0, the ellipticity at around 215 nm appears to be slightly different between the wild-type cathepsin E and the two mutants. Judging from the tight binding to the pepstatin A–Sepharose affinity column and the remaining enzymatic activity, however, the secondary and tertiary structures of the mutants seem to be essentially identical with those of wild-type cathepsin E, although this needs to be confirmed by X-ray or NMR analyses.

## DISCUSSION

It has been suggested that the propeptide in the ordinary pepsin family enzymes is held in place in the proenzyme cleft mostly by electrostatic interactions between its basic residues and acidic residues of the mature domain. Therefore, as in the case of ordinary aspartic proteinase zymogens, such as pepsinogen (*33, 34*) and cathepsin D (*35*), the propeptide of cathepsin E, which consists of 39 amino acids rich in basic residues (*8*), appears to interact electrostatically with the mature domain, which is abundant in acidic residues. Such an interaction is thought to be responsible for correct folding of the propeptide chain and control of the activation of the proenzyme. The proenzyme is known to be autoactivated when negatively charged residues become neutralized in an acidic environment, thereby resulting in proteolytic cleavage of the prosegment (*15, 32*). During the activation process, a large conformational change appears to be induced in the molecule by the removal of the propeptide. In addition, it is generally accepted that the two active-site Asp residues in animal members of the pepsin family work in concert to hydrolyze proteins: one, having a lower $pK_a$, mediates a deprotonated reaction; and the other, having a higher $pK_a$, acts as a proton donor. By analogy with pepsin, it has been proposed that one of the active-site Asp residues of cathepsin E deprotonates the nucleophilic water attacking the carbonyl carbon of the peptide bond, and the other protonates the carbonyl oxygen of the peptide bond, although the catalytic mechanism is currently unknown.

The present study showed that amino acid substitution at active-site residues strongly influences the enzymatic function. The single mutants of Asp-283 (D283A and D283E), as well as the double mutants (D98A/D283A and D98E/D283E), exhibited no proteolytic activity, and their proforms were not processed to the catalytically active enzymes under acidic conditions. On the other hand, while most of the amino acid substitutions for Asp-98 (D98A, D98A/D283A, and D98E/D283E) led to an inactive enzyme, the D98E mutant showed weak but significant enzyme activity toward both protein and synthetic substrates, and its zymogen was completely processed to the mature form under acidic conditions. The resulting mature mutant protein, like the wild-type mature cathepsin E, was resistant to further proteolysis. These results suggested that this mutant was correctly folded and had the autoprocessing activity, although the resultant mature protein had lower enzyme activity. When the catalytic properties of the D98E mutant were measured using acid-denatured hemoglobin and the synthetic substrate, the same trend for catalysis was observed for both substrates. These results indicate that the change in the catalytic properties by this mutation is not substrate-specific but could be attributed to the difference in the environments of the active site. Consistent with this view, the $K_m$ value of this mutant for the synthetic substrate was essentially the same as that of the wild-type cathepsin E, whereas the $k_{cat}$ value was significantly decreased, indicating that the mutation had no effect on substrate binding but affected overall efficiency of the catalysis. The CD spectra of the D98E mutant at various pH values were essentially identical with those of the wild-type cathepsin E, indicating that upon activation the mutant protein undergoes an irreversible change in spectrum into one that is indistinguishable from that of the wild-type enzyme. The above evidence clearly indicates that the Asp residue at position 98 can be replaced by the acidic residue Glu without complete loss of the enzyme activity, but that at position 283 can not. Thus, Asp-98 in cathepsin E is an essential but substitutable residue for the enzymatic activity, whereas Asp-283 is absolutely essential for catalysis.

It is noteworthy that the substitution of Thr at position 284 with Ser significantly affected the enzymatic function. This mutant was constructed as a mimic of BACE1, a novel transmembrane aspartic proteinase containing two active-site motifs (the DTG sequence in the N-terminal domain and the DSG sequence in the C-terminal domain). The hydroxy group in the Thr or Ser residues in the active-site motifs of aspartic proteinases is presumed to serve to create hydrogen bond(s) with a substrate in P1 and P2 positions, thereby stabilizing a transition state. Accordingly, since the T284 mutant retained the hydroxy group at the same position, its hydrogen bonding potential may results in catalytic properties similar to those of the wild-type

enzyme. Based on this assumption, the Thr residue at position 284 was replaced with Ser. Contrary to our expectations, the T284S mutant displayed a significant decrease in the enzyme activity while retaining the complete autoprocessing capability. The $K_m$ value of the T284S mutant was essentially the same as that of the wild-type enzyme, but the $k_{cat}$ value was significantly decreased by the mutation, indicating that the mutation had no effect on substrate binding but affected the overall efficiency of the catalysis. In particular, considering that the decrease in the $k_{cat}$ value reflects a significant change in the environment of the catalytic site of the mutant protein, it is most likely that the observed effect of this mutation is caused by the modification of the kinetic processes from the enzyme-substrate complex to the release of the product. Furthermore, it is important that the catalytic activities of this mutant were strongly inhibited by pepstatin A and the *Ascaris* pepsin inhibitor with the same $K_i$ values as those of the wild-type enzyme. These results suggest that the mutant has the correctly folded structure. This was further substantiated by the CD spectra of this mutant, which suggested that its secondary and tertiary structures are essentially identical with those of the wild-type enzyme. Therefore, it is logical to speculate that the presence of an additional $CH_2$ group in the T284S mutant created a different distribution of hydrogen bonds, which changed the enzyme-substrate interaction pattern and resulted in decreased catalytic rates. In this connection, it is interesting to refer to the recent finding by X-ray structural analysis that the active site of BACE1 is more open and less hydrophobic than those of other ordinary aspartic proteinases, although the bilobed structure is conserved (*36*). It has also been suggested that this open structure of BACE1 is mainly due to a unique insertion of 35 amino acids residues in the C-terminal lobe.

We concluded that the Thr residue in position 284 and two Asp residues in the active-site motifs are absolutely essential for the full catalytic activity of cathepsin E. To obtain a more definitive conclusion on the catalytic residues and mechanism of cathepsin E, further studies are necessary including X-ray crystallographic analysis of the three-dimensional structure of the enzyme.

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