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Biochemical Properties of the Monomeric Mutant of Human Cathepsin E Expressed in Chinese Hamster Ovary Cells: Comparison with Dimeric Forms of the Natural and Recombinant Cathepsin E

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Cathepsin E (CE) is the only known aspartic proteinase that exists as a homodimer consisting of two fully catalytically active monomers, which are covalently bound by a disulfide bond between two cysteine residues at the NH_2-terminal region (Cys^{43} in human pro-CE). To understand the physiological significance of the dimer formation, the monomeric mutant of human CE was constructed by site-directed mutagenesis ($Cys^{43} \rightarrow Ser^{43}$) and expressed in Chinese hamster ovary (CHO) cells. Immunolocalization of the mutant protein at both the light and electron microscopic levels revealed the monomeric CE to be associated predominantly with the endoplasmic reticulum and the non-lysosomal endocytic organelles. The cellular localization of the monomeric protein was compatible with that of the wild-type (dimeric form) of recombinant human CE expressed in the same cells. The monomeric protein was generated primarily as the 46-kDa pro-CE with a high-mannose-type oligosaccharide chain in the cells. In addition to the maximal activation at around pH 3.5, a substantial proportion of the monomeric pro-CE was converted to the mature form by incubation at pH 7 and 37°C for 5 min. In contrast, the dimeric pro-CE was scarcely activated by treatment at pH 7. Although catalytic properties of the *in vitro*-activated monomeric CE appeared to be indistinguishable from those of the dimeric forms of natural and recombinant CE, the monomeric form was more unstable to pH and temperature changes than these dimeric forms. These results indicate that the dimerization of CE is not necessarily required for proper folding to express activity, correct intracellular localization and carbohydrate modification, but that it may be essential to structurally stabilize the molecule *in vivo*.

Key words: aspartic proteinase, cathepsin D, cathepsin E, monomer mutant, structural stabilization.

Mammalian cells and tissues produce a number of aspartic proteinases, including secretory (*e.g.*, pepsin and renin) and nonsecretory proteins [*e.g.*, cathepsin D (CD) and cathepsin E (CE)]. Among these proteinases, CE has several distinctive features. (a) It is *N*-glycosylated with either the high-mannose-type or the complex-type oligosaccharide chain; the type of oligosaccharide chain appears to be cell-specific or to vary with cellular localization (*1-3*). (b) It is not found in lysosomes of any cell types under general conditions; its cellular localization varies with different cell types (*4*). (c) The tissue distribution of CE is rather limited; lymphoid tissues, gastrointestinal tracts, and urinary organs are particularly abundant in the enzyme (*5, 6*). (d) Activation of pro-CE occurs under acidic conditions predominantly through intermolecular reaction; the rate of activation was maximal at around pH 3.5 and greatly decreased at pH 2.0; this is different from that of the other aspartic proteinases, which are maximally activated at pH below 3.5 (*e.g.*, pH 2.0 and below for pepsinogens) (*1, 3, 7*).

In addition, CE appears to be unique in existing as a homodimer of two fully catalytically active monomers (79-90-kDa) in a native state (*7, 8*). The other aspartic proteinases are primarily present as monomeric enzymes with a molecular mass of about 40-kDa (*9*). The two identical subunits of CE are connected by a disulfide bond between a cysteine residue at the N-terminal of their prosegments (Cys^{43} in human, rabbit, and rat; numbering is based on the sequence of human pro-CE) (*8*). We previously reported that the conversion of the dimeric CE to the monomeric

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Abbreviations: CD, cathepsin D; CE, cathepsin E; CHO, Chinese hamster ovary; Con A, concanavalin A; dhfr, dihydrofolate reductase; ER, endoplasmic reticulum; H297, Pro-Thr-Glu-Phe(CH_2-NH)-Nle-Arg-Leu; L363,564, Boc-His-Pro-Phe-His-Sta-Leu-Phe-NH_2; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TRAPα, translocon-associated protein α subunit; Z-Arg-Arg-MCA, benzyloxy-carbonyl-arginyl-arginine-4-methyl-7-coumarylamide.

form occurred in the presence of a reducing agent without a loss of the activity (*8*). Recent studies have also shown that interconversion between the dimeric and monomeric pro-CE occurs reversibly depending on the oxidation-reduction potential and that the monomeric CE is unstable at weakly alkaline pH (*7, 10, 11*). On the other hand, our previous results revealed that the wild type of recombinant human CE expressed in CHO cells was localized to the endoplasmic reticulum (ER), the vacuole-like structures, and the soluble cytosolic compartment (*12*). This localization appears to be compatible with those of endogenous CE in other cell types (*4, 13-16*) or transfected CE in monkey Cos 1 cells (*17*).

These characteristic features of CE have raised fundamental questions about the physiological significance of CE as a dimeric proteinase. What are the differences in catalytic and physicochemical properties between the monomeric and dimeric CE? Is CE actually present in the monomeric form *in vivo*? If so, when and where is the monomeric form generated? To pursue these issues, we expressed the monomeric mutant of human CE (the Cys^{43} →Ser construct) in CHO cells and studied its intracellular localization and catalytic and physicochemical properties, as compared with those of the dimeric forms of natural and recombinant human CE.

EXPERIMENTAL PROCEDURES

Materials—Antisera against CE purified from human erythrocyte membranes (*18*) and CD purified from rat spleen (*19*) were raised in rabbits and purified by affinity chromatography as described previously (*20, 21*). Antiserum against translocon-associated protein α subunit (TRAPα) was kindly provided by Dr. T.A. Rapoport (Harvard Medical School, MA) and M. Wiedmann (Sloan-Kettering Cancer Center, NY). Pro-Thr-Glu-Phe(CH_2-NH)Nle-Arg-Leu (H297) was generously provided by Dr. M. Szelke (Ferring Research Institute, University of Southampton, UK). Boc-His-Pro-Phe-His-Sta-Leu-Phe-NH_2 (L363,564) was kindly supplied by Dr. J. Boger and Dr. J. Jacobs (Merk, Sharp, and Dohme Research Laboratories, USA). Endoglycosidase H was purchased from Seikagaku Kogyo (Tokyo). Benzyloxy-carbonyl-arginyl-arginine-4-methyl-7-coumarylamide (Z-Arg-Arg-MCA) were from the Protein Research Foundation (Osaka). Mutant-K kit was from Takara Shuzo, Japan. All other chemicals were of reagent grade and were purchased from various commercial sources.

Construction of Expression Mutant Plasmid—The wild-type CE cDNA expression plasmid, pCE-dhfr (*12*), was digested with *Bam*HI and the resulting 1.1 kb fragment was subcloned in the *Bam*HI site of pUC118 (pUC118 CE). Oligonucleotide 5′-CCGAGTCCTCTTCAATGGACC-3′ containing a *Mbo*II site (TCTTC) was used for introduction of Cys^{43} to Ser^{43} amino-acid substitution (the underline indicates noncomplementary nucleotides). Mutagenesis was performed according to the procedure of Kunkel (*22*) using a Mutant-K kit. The clones containing the desired mutation were screened by *Mbo*II restriction analysis and the mutation was confirmed by DNA sequencing based on the dideoxy nucleotide chain termination method (*23*). The 1.1-kb *Bam*HI fragment of the pUC118 CE, encoding the introduced mutation, was isolated and ligated with the 4.6-kb *Bam*HI fragment of the pCE-dhfr to generate the expression plasmid pCE-C43S-dhfr (Fig. 1). Transfection and selection were performed as described previously (*12*).

Enzyme Assays and Protein Determination—Acid proteinase activity was determined at pH 3.8 using 1.5% acid-denatured hemoglobin as a substrate as described previously (*18*). The levels of CE and CD were determined by quantitative immunoprecipitation using discriminative antibodies specific for each enzyme, as described previously (*6*). In experiments to determine the effect of ATP on the CE activity at pH 5.5, the fluorescamine assay was performed using 0.1% hemoglobin as a substrate as described previously (*1*). Cathepsin B was assayed with Z-Arg-Arg-MCA as a substrate according to the method of Barrett and Kirschke (*24*), with some modifications (*25*). β-Glucuronidase was assayed with 4-methylumbelliferyl-β-D-glucuronide as a substrate by the method of Robins *et al.* (*26*). Protein was determined by the method of Lowry *et al.* (*27*) with bovine serum albumin as a standard.

Immunofluorescense Microscopy—Cells grown on glass coverslips were washed with phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde in PBS containing 0.1% glutaraldehyde, and 0.5% dimethyl sulfoxide for 1 h at 4°C. After washing with PBS, the cells were incubated with 1% bovine serum albumin in PBS containing 0.1 M lysine for 1 h on ice, and then with primary antibodies in PBS for 2 h at 4°C. After washing to remove unbound antibodies, the cells were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA). The cells were washed with PBS and stained with a streptavidin-fluorescein isothiocyanate conjugate (Life Technology, Gaithersburg, MD) followed by washing in PBS. The cells were viewed in a Leitz DMRB/E fluorescence microscope (Leica, Switzerland). Primary antibodies used were the monospecific anti-human CE IgG, the monospecific anti-rat CD IgG as a marker for lysosomes, and TRAPα as a marker for the ER.

Immunoelectron Microscopy—Confluent cells were washed with PBS and fixed with 3% formaldehyde in 0.1 M sodium phosphate buffer containing 0.3% glutaraldehyde and 0.05% $CaCl_2$ at pH 7.4. After washing with 0.1 M sodium phosphate buffer, pH 7.4, the fixed cells were dehydrated in a graded series of ethanol, and then embedded in Lowicryl K4M (Polaron, Watford, Herts, UK). The labeling procedures were essentially based on the method described by Goto *et al.* (*28*). Ultrathin sections were mounted on nickel grids coated with collodion and floated on PBS containing 1% bovine serum albumin and 0.1 M lysine-HCl for 10 min at room temperature. The sections were then incubated with the monospecific anti-human CE IgG (50 μg/ml) for 1 h at room temperature. After repeated rinsing in PBS, the grids were incubated with colloidal gold-labeled goat anti-rabbit IgG (gold particles 15 nm in diameter, 1:20 dilution) for 20 min at room temperature. The grids were then washed three times for 5 min and stained with 2% uranyl acetate, then examined with a Hitachi H-7000 electron microscope. As controls, nonimmune rabbit IgG was used as primary antibodies.

Subcellular Fractionation and Percoll Density Gradient Centrifugation—The transfected CHO cells were grown to confluency, washed three times with PBS, and dispersed with the buffer containing 0.25% trypsin. Subcellular fractionation and Percoll density gradient centrifugation

were performed essentially under the same conditions as previously employed for those of the dimeric CE from the transfected CHO cells (*12*). Briefly, after being harvested by centrifugation, the cells were suspended in PBS at a density of 2×10^7 cells/ml and disrupted by nitrogen cavitation for 20 min at 400 psi in a bomb (Parr Instrument, Moline, IL) at 4°C. After centrifugation at $400\times g$ for 8 min to remove nuclei and unbroken cells, 2 ml of the postnuclear supernatant was layered on 5.5 ml of the iso-osmotic Percoll solution (27% Percoll/0.25 M sucrose/2 mM $MgCl_2$/5 mM Tris-HCl, pH 7.4). One milliliter of the dense sucrose solution (2.5 M sucrose/2 mM $MgCl_2$/5 mM Tris-HCl, pH 7.4) was placed at the bottom of the tubes. The tubes were centrifuged at $39,000\times g$ for 40 min in a Hitachi 65 rotor and fractionated from the top into 15 fractions of 0.5 ml with a glass syringe.

Gel Electrophoresis and Immunoblots—Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed following the procedure of Laemmli (*29*). For immunoblotting, proteins electrophoresed on SDS-gels were transferred electrophoretically at 100 V for 12-15 h from the gels to nitrocellulose membranes according to the method of Towbin *et al.* (*30*). The blotted membranes were immunostained as described previously (*26*).

Conversion of Procathepsin E into the Mature Enzyme—Conversion of the recombinant pro-CE was carried out by incubation at 37°C for 5 min at pH 3.5 and 7.0. After incubation, the activation was stopped by addition of 200 mM Tris-HCl buffer, pH 9.0, to give a final concentration of 20 mM. The samples were analyzed by SDS-PAGE followed by immunoblotting.

Treatment of Endoglycosidase H—The purified enzyme samples were dissociated by boiling for 2 min with 10 μl of 1% SDS in water. To these samples, 90 μl of 50 mM sodium acetate buffer, pH 6.0, containing endoglycosidase H (10 mU), pepstatin (25 μg/ml), leupeptin (25 μg/ml), chymostatin (25 μg/ml), phenylmethylsulfonyl fluoride (85 μg/ml), 0.05% SDS, and 0.75% Triton X-100 was added. The reaction mixtures were incubated for 18 h at 37°C, then analyzed by SDS-PAGE followed by immunoblotting.

RESULTS

Design and Description of the Monomeric Mutant of Human CE—The construction scheme of the monomeric human CE expression plasmid is illustrated in Fig. 1. The monomeric mutant was designed by Cys^{43} to Ser^{43} amino acid substitution in the wild-type CE cDNA expression plasmid pCE-dhfr to generate the expression plasmid pCE-C43S-dhfr. After introducing either pCE-C43S-dhfr or pCE-dhfr into dihydrofolate reductase-deficient CHO cells (CHO $dhfr^-$), the transfected cells selected for dihydrofolate reductase-positive phenotype (CHO $dhfr^+$) were cloned and subsequently screened for expression of CE. Immunoblotting analysis of cell extracts revealed that the cloned CHO $dhfr^+$ cells contained proteins reactive with anti-human CE IgG (not shown). The expression level of the mutant protein, as judged from the intensity of the staining with anti-human CE IgG, was similar to that of the dimeric form of CE (Fig. 2).

Distribution and Cellular Localization of Monomeric CE in Transfected CHO Cells—The intracellular distribution of the monomeric mutant of human CE was monitored by indirect immunofluorescence staining of the transfected cells. In control experiments on untransfected cells or on cells transfected with the vector alone, no fluorescence signal was detected. As shown in Fig. 2, the cells expressing the CE monomer displayed punctate fluorescence over the whole cytoplasm. This pattern appeared to be characteristic of vesicular protein expression. In addition, the cells showed the perinuclear, reticulate fluorescent staining characteristic of proteins retained in the ER. The reticulate staining of monomeric CE appeared similar to the diffuse reticulum pattern labeled by the antiserum to TRAPα, a ER-specific component previously called "signal sequence receptor" (*31*). In contrast, the punctate staining of monomeric CE appeared distinct from the granular staining of lysosomal CD. These staining patterns of monomeric CE were similar to those of the dimeric form of human CE

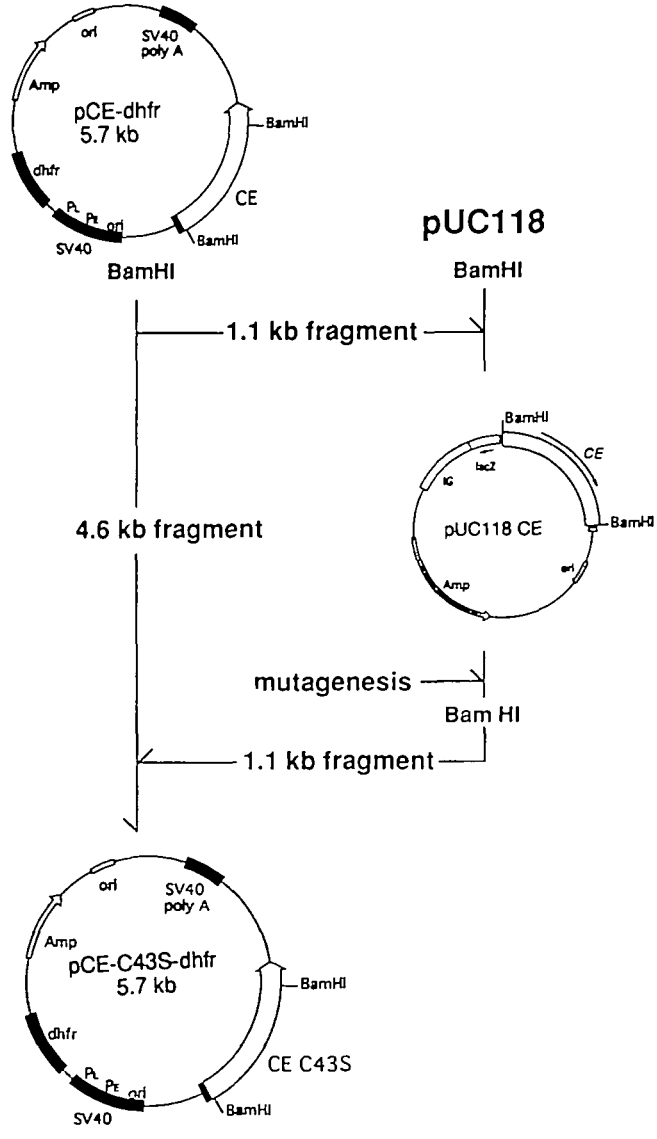

Fig. 1. **Diagram of the construction of the expression plasmid of the monomeric form of human CE.** dhfr, mouse dihydrofolate reductase gene; Amp, β-lactamase gene from pBR322; poly(A), the polyadenylation signal from SV40; CE, human preprocathepsin E cDNA; SV40, SV40 early promoter.

expressed in CHO cells, indicating that the mutation produces no significant change in its cellular localization. The intracellular localization of monomeric CE was further examined at the electron microscopic level by immunolabeling ultrathin sections of the transfected cells. Labeling for monomeric CE were predominantly associated with the ER and the vacuole-like structures (Fig. 3). Significant labeling was also localized on non-membranous reticular structures. Whether this simply reflects labeling of the cytoplasmic matrix or of the protein dissociated from the organelles during preparation is presently unclear. Labeling was rarely observed on mitochondria, lysosomes, or within nuclei. No immunoreaction was detected in control experiments with non-immune IgG. These results suggest that the dimer formation of CE is not necessarily essential for intracellular localization of the molecule.

Further support for the distribution of monomeric CE into these two distinct cellular compartments was obtained from subcellular fractionation by centrifugation in Percoll gradients. When the postnuclear supernatant obtained by centrifugation of the disrupted cells at $400 \times g$ for 8 min was subjected to Percoll density gradient centrifugation, the monomeric CE was separated into two pools; the light and the high-density pools equilibrated in the density range of 1.006-1.025 (g/ml) and 1.095-1.125 (g/ml), respectively (Fig. 4).

Purification of the Monomer CE—Monomeric CE was purified from the transfected CHO cells essentially according to the procedure as described (*12*). Briefly, the two enzyme-active pools (the light and high-density pools obtained by Percoll density gradient centrifugation) were centrifuged at $105,000 \times g$ for 60 min to remove Percoll and then ultrasonicated in the presence of 0.5% Triton X-100. Each supernatant was separately applied to Con A-Sepharose columns equilibrated with 20 mM sodium phosphate buffer, pH 7.0, containing 0.05% Brij 35 and 1 M NaCl. Each column was washed with the same buffer, and the enzyme activity was eluted with 0.5 M methyl-α-D-mannoside in the buffer. After dialysis against 10 mM sodium phosphate buffer, pH 7.0, the concentrated enzyme solution was subjected to anion-exchange chromatography on a Mono Q column which had been equilibrated with the same buffer and eluted by the step-wise method (Fig. 5). The pooled active fractions were each concentrated and run on a gel filtration column of TSK-Gel G2000SW in the FPLC system, equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 100 mM Na_2SO_4. When the final preparations were analyzed by SDS-PAGE under nonreducing conditions, each produced a single protein band with an apparent molecular mass of 46-kDa (Fig. 6). A single protein band was also observed with the same molecular mass under reducing conditions (not shown). The results indicate the homogeneity of each preparation. On the other hand, SDS-PAGE of the natural human CE from erythrocyte membranes under nonreducing conditions revealed a single protein band at the position corresponding to an apparent molecular mass of 82-kDa. A summary of the purification is shown in Table I.

Acid Treatment and Endoglycosidase H Digestion—When the monomeric forms of human CE purified from both the light- and the high-density pools, called s-CEm and v-CEm, respectively, were incubated in 10 mM sodium acetate buffer, pH 3.5, at 37°C for 5 min followed by SDS-PAGE and immunoblotting, the 46-kDa proteins were completely converted into the mature forms with an apparent molecular mass of 42-kDa (Fig. 7A). A substantial proportion of the 46-kDa proteins was also converted to the mature forms by incubation at 37°C for 5 min in 10 mM sodium phosphate buffer, pH 7.0. This conversion was inhibited by pepstatin (10 μM), a specific inhibitor of aspartic proteinases (not shown). The susceptibility to conversion to the mature form showed a strong contrast with the resistance to conversion of the dimeric forms of natural (*3*) and recombinant pro-CE (*12*) to the mature

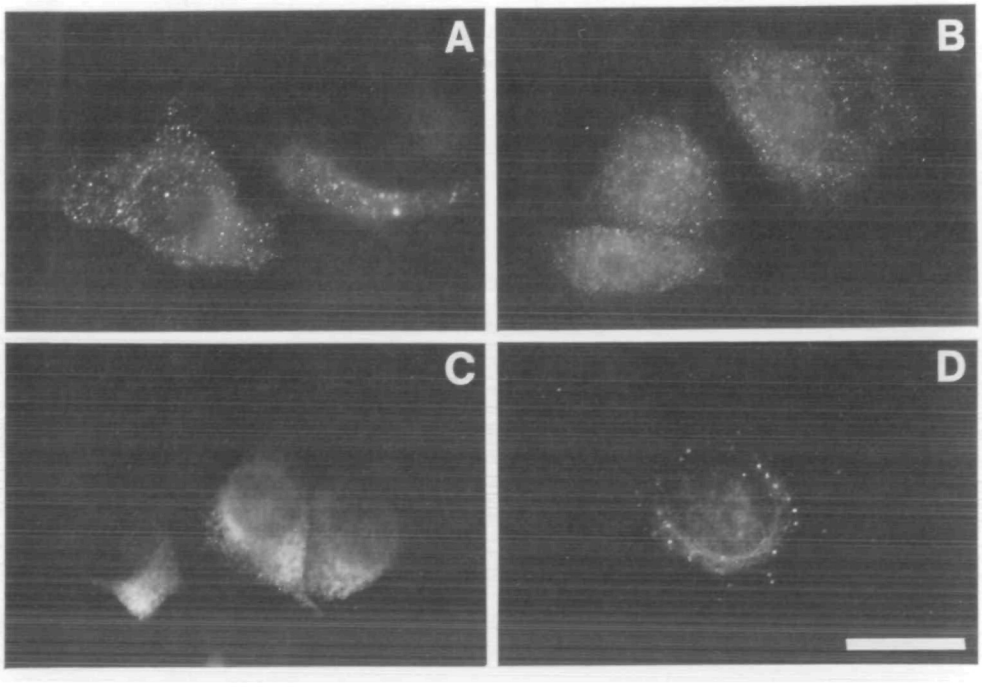

Fig 2. **Immunofluorescence microscopy of CHO cells transfected with plasmid constructs encoding the monomeric (A, C, D) and dimeric (B) forms of CE.** The transfected cells were fixed and stained with affinity-purified anti-human CE IgG (A, B), anti-TRAPα IgG (C), and affinity-purified anti-rat CD IgG (D) as described under "EXPERIMENTAL PROCEDURES." Punctate and reticular stainings of monomeric CE were seen Bar 25 μm.

forms at pH 7.0. The mature (dimeric) form of natural human CE purified from the erythrocyte membrane did not undergo any alternation when analyzed under the same conditions. These results indicate that both s-CEm and v-CEm are pro-CE and that they are more susceptible to autocatalytic proteolysis at neutral pH than the dimeric forms.

To examine the type of carbohydrate moieties, deglycosylation of both s-CEm and v-CEm was performed by using endoglycosidase H, which is specific for high-mannose-type oligosaccharide chains and digests between the two proximal *N*-acetylglucosamine residues of asparagine-linked carbohydrate chains, and the resulting digests were analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 7B, both the 46- and the 42-kDa forms from each pool were found to be sensitive to endoglycosidase H, indicating that all of the monomeric forms are *N*-glycosylated with high-mannose-type oligosaccharide chains.

Enzymatic Properties—Enzymatic properties of the monomeric forms of CE were compared with those of the dimeric forms of natural and recombinant human CE. Since pro-CE is known to be catalytically inactive and to be rapidly activated to the mature enzyme under acidic conditions at around pH 3.5 (*1*), catalytic properties of the purified monomeric CE were analyzed as the mature forms

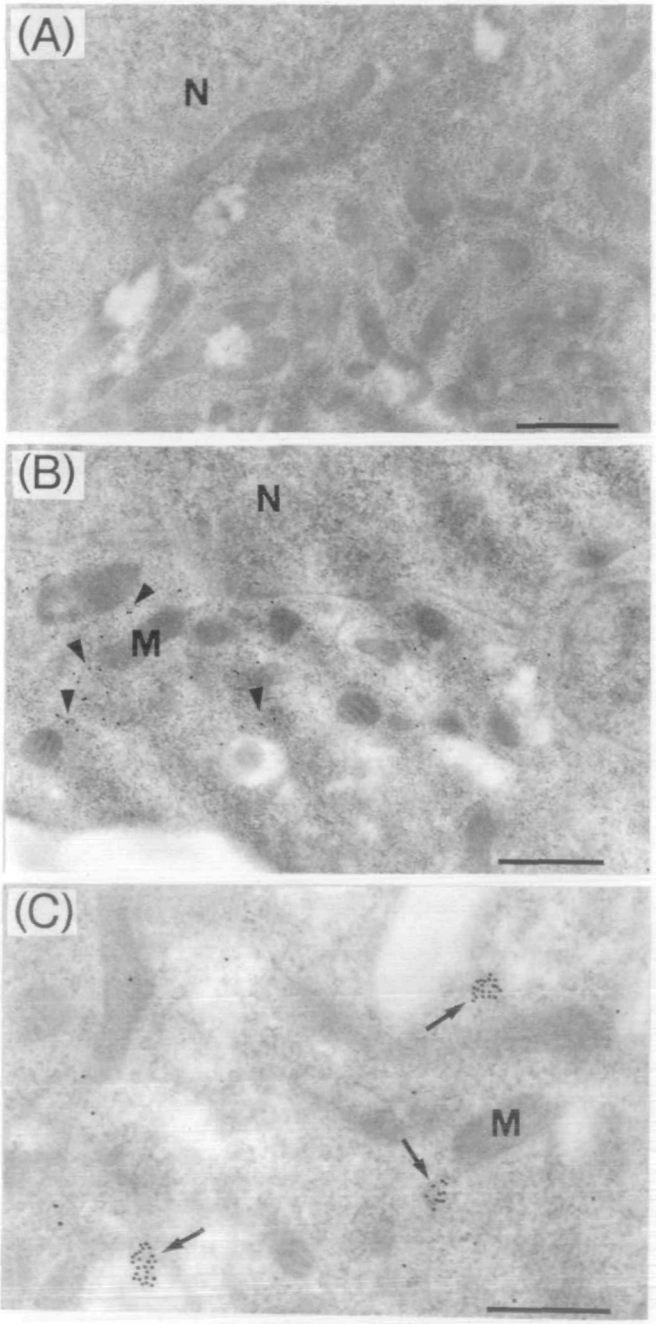

Fig. 3. **Immunoelectron microscopic localization of the monomeric mutant of CE.** No immunoreactivity was observed with nonimmune rabbit IgG (A). Panels B and C are from different fields of the immunostained transfected cells. Gold particles indicating the antigenicity of the monomeric CE were clearly associated with the ER (arrow heads) and the vacuole-like structures (arrows). Small amounts of gold particles were also found in the cytoplasmic matrix. The labeling was rarely observed on the nuclei and mitochondria. N, nucleus; M, mitochondria. Bars 0.5 μm.

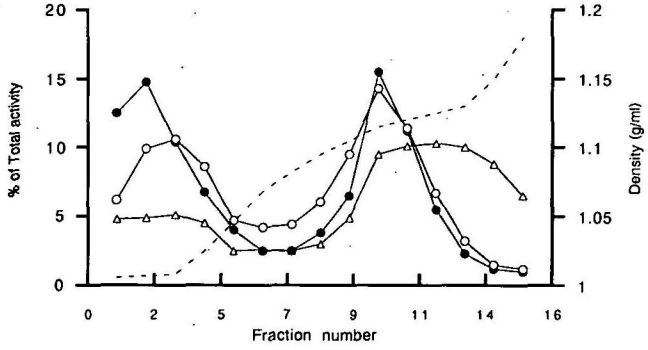

Fig. 4. **Distributions in Percoll density gradients of the monomeric CE, cathepsin B, and β-glucuronidase in postnuclear supernatant fractions of CHO cells.** The transfected CHO cells were disrupted by nitrogen cavitation and centrifuged. The postnuclear supernatant fraction was subjected to Percoll density gradient centrifugation as described under "EXPERIMENTAL PROCEDURES." Gradients were collected in 15 fractions of 0.5 ml and analyzed for activities of CE (●), β-glucuronidase (○), cathepsin B (△), and density (dashed line).

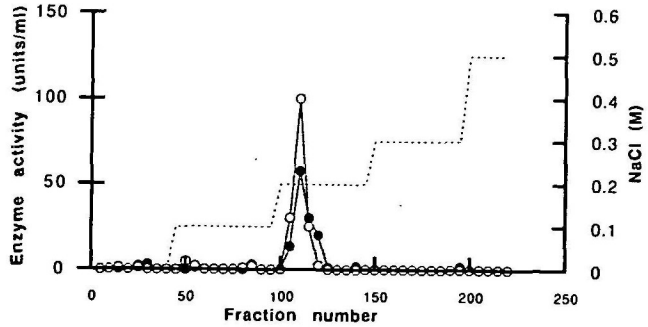

Fig. 5. **Anion-exchange chromatography on a Mono Q column of the monomeric CE fractions (s-CEm and v-CEm) obtained by Con A-Sepharose affinity chromatography.** The enzyme solutions of s-CEm (●) and v-CEm (○), which had been separated by Percoll density gradient centrifugation followed by Con A-Sepharose chromatography, were each applied to a Mono Q column equilibrated with 10 mM sodium phosphate buffer, pH 7.0. The flow rate was 0.5 ml/min and fractions of 2 ml were collected. ·····, NaCl concentration.

activated during the assay. The specific activities of both purified s-CEm and v-CEm against acid-denatured hemoglobin were comparable to those of the dimeric forms of recombinant s-CE and v-CE, as described (*12*). Pepstatin was the most potent inhibitor of both s-CEm and v-CEm and the K_i values for the monomeric forms were very similar to those for the dimeric forms of natural and recombinant CE (Table II). The two synthetic inhibitors, Pro-Thr-Glu-Phe(CH_2-NH)Nle-Arg-Leu (H297) and Boc-His-Pro-Phe-His-Sta-Leu-Phe-NH_2 (L363,564), were also potent inhibitors of all of the monomeric and dimeric enzymes. There was no significant difference in K_i values for each inhibitor between s-CEm and v-CEm and between the monomeric and dimeric forms. Also, there was no significant difference in optimal pH for digestion of various proteins and synthetic substrates and substrate specificity between the monomeric and dimeric forms (data not shown). These results strongly suggest that the catalytic properties of the monomeric forms are essentially identical with those of the dimeric forms.

Physicochemical Properties—The pH stability of s-CEm and v-CEm was investigated by preincubation for 1 h at 37°C in a variety of buffers in the range of pH 4–9 and compared with those of the dimeric forms of natural and

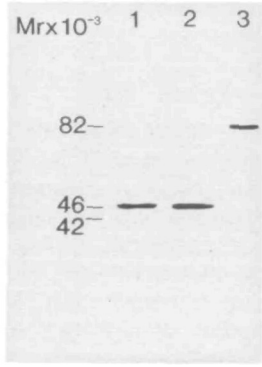

Fig. 6. **SDS-PAGE under nonreducing conditions of the purified monomeric CE.** SDS-PAGE was performed with 10% polyacrylamide gels in Tris-HCl buffer, pH 8.9, under nonreducing conditions. The final preparations of s-CEm and v-CEm were subjected to SDS-PAGE after denaturation at 37°C for 30 min in 1% SDS. Lane 1, s-CEm; lane 2, v-CEm; lane 3, human erythrocyte membrane CE. After electrophoresis, the polypeptides were visualized by the silver stain.

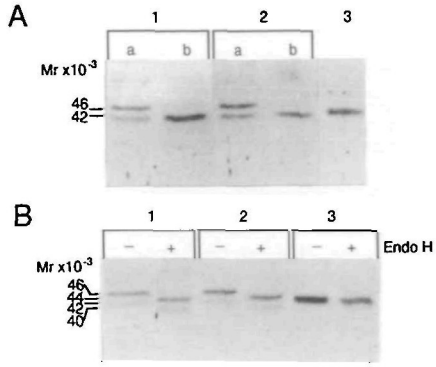

Fig. 7. **(A) Effect of incubation pH on the monomeric pro-CE conversion to the mature form.** Samples of the purified monomeric CE were incubated at 37°C for 5 min at pH 7.0 (a) or pH 3.5 (b). As a control, the mature form of human erythrocyte CE was treated at pH 3.5 and 37°C for 5 min (lane 3). After incubation at 37°C for 30 min in 1% SDS, each sample was analyzed by SDS-PAGE under reducing conditions followed by immunoblotting. Lane 1, s-CEm; lane 2, v-CEm; lane 3, human erythrocyte CE. **(B) Sensitivity of the monomeric forms of pro-CE and the mature form of human erythrocyte CE to endoglycosidase H treatment.** The purified samples were incubated at 37°C for 18 h with or without endoglycosidase H followed by SDS-PAGE under reducing conditions and immunoblotting. Lane 1, s-CEm; lane 2, v-CEm; lane 3, human erythrocyte CE.

TABLE II. ***Kinetic constants (K_i) for the inhibition of monomeric and dimeric forms of recombinant CE compared with those of natural CE.***

	Pepstatin (nM)	H297[a] (nM)	L363,564[b] (nM)
s-CEm	0.073	8.3	6.4
v-CEm	0.080	16.2	6.5
s-CE	0.070	11.6	4.6
v-CE	0.073	7.8	7.5
Natural CE	0.066	15.8	14.5

[a]H297: Pro-Thr-Glu-Phe(CH_2-NH)Nle-Arg-Leu, [b]L363,564: Boc-His-Pro-Phe-His-Sta-Leu-Phe-NH_2.

TABLE I. ***Purification of the recombinant monomer-CE expressed in CHO cells.*** The CE activity was determined at pH 3.8 using acid-denatured hemoglobin as a substrate after immunoprecipitation with the anti-human CE antibody as described in "EXPERIMENTAL PROCEDURES." One unit is defined as the amount of enzyme which gives an absorbance at 660 nm equivalent to 1 mg of tyrosine in 1 min.

Purification	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Homogenate	210	13,200	63	100	1
Postnuclear supernatant	162	11,600	73	88	1.1
Percoll fractionation					
s-CEm	–	5,000	–	38	–
v-CEm	–	7,100	–	36	–
Con A-Sepharose					
s-CEm	1.5	3,750	2,500	28	40
v-CEm	4.7	8,300	1,990	38	32
Mono Q					
s-CEm	0.11	1,650	15,000	13	239
v-CEm	0.12	2,100	16,750	15	267
TSK-Gel G2000SW					
s-CEm	0.047	1,240	22,500	13	453
v-CEm	0.051	1,750	36,000	14	573

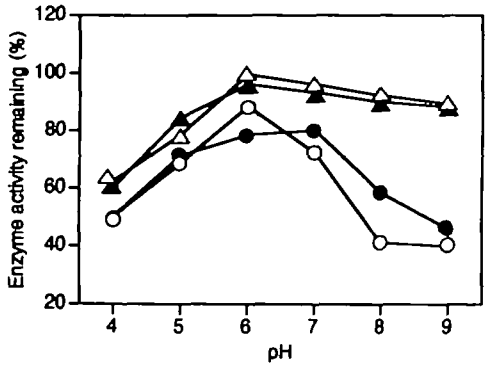

Fig. 8. **Effect of pH on activities of the monomeric and dimeric forms of recombinant CE purified from the transfected CHO cells.** The samples were preincubated at 37°C for 1 h at various pH values, then the remaining activity was determined at pH 3.8 using 1.5% acid-denatured hemoglobin as a substrate. Buffers used were 0.1 M sodium acetate (pH 4 and 5), 0.1 M sodium phosphate (pH 6, 7, and 8), and 0.1 M Tris-HCl (pH 9.0). Monomeric forms: (●, s-CEm; ○, v-CEm), dimeric forms (▲, s-CE; △, v-CE).

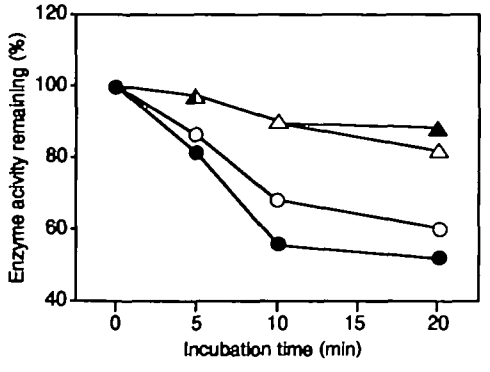

Fig. 9. **Effect of incubation at 55°C and pH 5.5 on the stability of the monomeric and dimeric forms of recombinant CE.** The purified samples were preincubated at 55°C and pH 5.5. At the indicated times, the samples were collected and the remaining activity was determined. Monomeric forms: (●, s-CEm; ○, v-CEm); dimeric forms: (▲, s-CE; △, v-CE).

recombinant CE. As shown in Fig. 8, both the monomeric and dimeric forms showed the significant instability at pH 4-5, probably due to their autodegradation, although the monomeric forms were apparently the less stable of the two. A marked difference between the monomeric and dimeric forms was observed at above pH 7. The dimeric forms were stable at above pH 5, whereas the monomeric forms were greatly inactivated in the range of at pH 7-9. The dimeric forms of natural and recombinant CE were indistinguishable in pH stability over the range of pH 4-9.

When the effect of preincubation at 55°C and pH 5.5 on the activity was investigated, the monomeric forms were apparently less stable than the recombinant dimeric forms (Fig. 9). After 10 min of incubation, the activities of s- and v-CEm fell to about 55 and 70%, respectively, of those measured with the respective untreated forms, whereas the recombinant dimeric forms (both s- and v-CE) retained more than 90% of their initial activities. The results indicate that the dimeric forms have greater thermal stability than the monomeric forms.

In previous studies, human erythrocyte CE has been

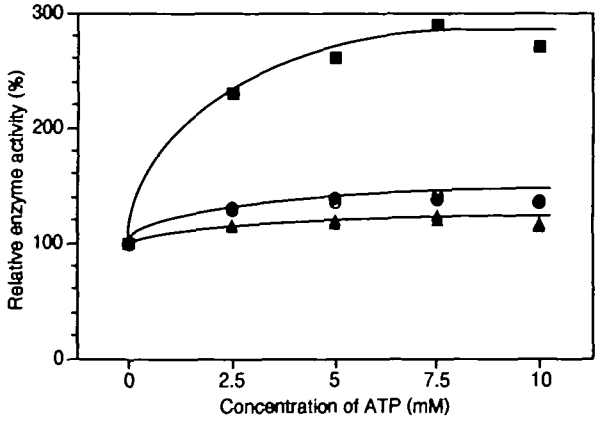

Fig. 10. **Effects of ATP on the hydrolysis of hemoglobin by natural and recombinant CE.** Aliquots of each enzyme sample in 0.1 M sodium acetate buffer (pH 5.5) were preincubated at 40°C for 40 min with or without various concentrations of ATP, then added to the protein substrate solution (final 0.1% hemoglobin) in the same buffer. The reaction was stopped by addition of 5% trichloroacetic acid, and acid-soluble products were determined by the fluorescamine method as described under "EXPERIMENTAL PROCEDURE." Recombinant monomeric forms: (●, s-CEm; ○, v-CEm), recombinant dimeric forms (▲, s-CE; △, v-CE), and human erythrocyte CE (■).

shown to be activated by ATP (*1, 32*) and RNA (*33*) and to be restored to virtually full activity at pH 5.8, at which the enzyme is essentially inactive by itself. This is considered to be due to the stabilization of the enzyme by these compounds in such a way as to maintain its active conformation. Therefore, the effect of ATP on the monomeric and dimeric forms was examined at pH 5.5. As shown in Fig. 10, ATP activated human erythrocyte CE (the natural dimer) by up to about 280% of the initial activity measured in its absence, whereas it scarcely activated the recombinant monomeric and dimeric forms. Since human erythrocyte CE has a complex-type carbohydrate chain (*1*) and since the monomeric and dimeric forms of recombinant human CE are glycosylated with high-mannose-type oligosaccharide chains, the difference in susceptibility to ATP activation between the natural and recombinant proteins may be due to the difference in their carbohydrate moieties.

DISCUSSION

To study the physiological significance of the existence of CE as a disulfide-linked homodimer, we decided to prepare heterologous cells expressing the monomeric form of CE. In this paper, we report the high level expression and intracellular localization of the monomeric mutant of human CE in CHO cells. Biochemical studies combined with fluorescence and electron microscopy have indicated that the monomeric CE is mainly associated with the ER and the vacuole-like structures. Further, only a small portion of the monomeric enzyme was found to be localized in the soluble cytosolic compartment, as revealed by the high speed centrifugation (105,000×*g* for 60 min) of the light density pool of the post-nuclear supernatant of the cells, which is obtained by Percoll density gradient fractionation and composed of the ER and soluble cytosolic components. These data are in accordance with those obtained with the wild type of recombinant human CE expressed in CHO cells (*12*).

However, it is still difficult to completely rule out the possibility that the cytosolic localization of monomeric CE may be artificially produced in the course of subcellular fractionation or during preparation of specimens for immunoelectron microscopy. The monomeric forms in the cells were likely to exist exclusively as a 46-kDa proenzyme with a high-mannose-type oligosaccharide chain, since the 42-kDa mature form was barely detectable in the cell extract of the transfected cells, as revealed by immunoblot analysis (not shown). This was further supported by pulse-chase analysis of CE synthesis with [^{35}S]methionine, in which the production of the 42-kDa mature form of monomeric CE seemed unlikely to occur during the chase up to 96 h (Tsukuba *et al.*, unpublished). Therefore, it is unlikely that the monomeric forms associated with the ER and the vacuole-like structures have a precursor-product relationship, although the role of the endogeneous processing machinery in the existence of different intracellular localization remains to be established. The localization of monomeric CE in distinct cellular compartments, somewhat analogous to that of the wild type of recombinant CE (*12*), may reflect the distinct intracellular pools which can function in response to different cellular stimuli. More recently, Finley and Kornfeld (*17*) reported that the wild-type recombinant human CE transfected in mouse L cell fibroblasts and monkey Cos 1 kidney cells was localized primarily in the ER, and they suggested that the ER retention might be due to its interaction with the putative ER-binding protein. They further suggested that the high level expression of CE might cause the saturation of the putative ER-binding protein and its release from the ER. Based on systematic analyses of various constructs for chimeric (between human CE and pepsinogen) and mutant (Cys^{43}→Ala) proteins expressed in Cos 1 cells, these arthors also suggested that the first 48 amino acid residues of the mature enzyme, which include the 43rd cysteine residue, are important for specific retention of CE in the ER.

Although both the monomeric and the dimeric forms exhibited similar intracellular localization in the transfected cells, they appeared to undergo different post-translational modifications. As described above, the monomeric forms existed exclusively in the 46-kDa proform and their mature forms were barely detectable, whereas the dimeric forms were found predominantly in a 90-kDa proform and an 84-kDa intermediate form, and present slightly but clearly as an 82-kDa mature form (*12*). The dimerization, therefore, may provide the proper tertiary structure to undergo proteolytic processing. However, as both the monomeric and dimeric forms are *N*-glycosylated with high-mannose-type oligosaccharides, the carbohydrate modification appears to be scarcely influenced by the monomer formation. Upon a brief acid treatment, the monomeric pro-CE was rapidly converted to the mature forms. This conversion was very similar to that of the dimeric forms of natural (*1, 3, 7*) and recombinant CE (*12*). However, there was a significant difference in the rate or extent of activation of the two forms when treated at neutral pH. While the dimeric forms were scarcely converted to the mature forms by incubation at pH 6-7 (*3, 7*), considerable amounts of the monomeric forms were converted to the mature forms under the same conditions. Since the activation reaction involves autocatalytic proteolysis and cleavage of the activation segment from the proenzyme (*1, 34*), and since the intramolecular reaction is thought to be important in the initial phase of activation to produce the active enzyme (*1, 3*), the difference in activation rate between the two forms appears to be due to the difference in susceptibility to autocatalytic proteolysis at neutral pH. Taken together, the active forms are shown to be more unstable than the inactive forms, which is due to autodegradation under acidic conditions close to optimum pH (Fig. 8) (*7, 35*). Therefore, it is more likely that the ease of activation of the monomeric forms at neutral pH implies their structural instability.

No significant difference in catalytic properties was observed between the *in vitro*-activated monomeric and dimeric forms. However, there were distinct differences between the two forms with regard to pH and thermal stability. The monomer forms were more unstable than the dimeric forms of recombinant CE at above pH 7. Similar results have been obtained with the monomeric forms of guinea pig CE and pro-CE reduced by 2-mercaptoethanol (*7, 35*) and the nonglycosylated, monomeric mutant of human CE (the Cys^{43}→Ala construct) expressed in *Escherichia coli* (*11*). The susceptibility of the monomeric forms to heat inactivation was higher than that of the dimeric forms. The pH and thermal stability of naturally occurring human CE was similar to that of the dimeric forms of recombinant CE (not shown). In addition, it was found that the monomeric CE produced in the presence of 2-mercaptoethanol (*35*) and the Cys^{43}→Ala mutant (*11*), as compared to the dimeric enzyme, had a markedly reduced half-life at alkaline pH. In the light of these findings, the present data strongly suggest that the monomeric forms are more susceptible to conformational changes leading to inactivation. Therefore, it is more likely that the monomeric forms are in the process of degradation.

In conclusion, the present study clearly demonstrates that the dimerization of CE is not necessarily required for proper folding to express activity, correct intracellular localization, and carbohydrate modification, but may be essential to structurally stabilize the molecule *in vivo*.

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