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Identification of the Catalytic Triad Residues of Porcine Liver Acylamino Acid-Releasing Enzyme

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Acylamino acid-releasing enzyme (AARE) [EC 3.4.19.1] is a tetrameric serine protease, which belongs to the oligopeptidase family and specifically removes acetyl amino acids from N-terminally acetylated peptides. By using diisopropyl fluorophosphate, we previously identified one of the residues comprising the catalytic triad of this enzyme as Ser^{587} [Miyagi, M. *et al.* (1995) *J. Biochem.* 118, 771-779]. To elucidate the other two residues forming the catalytic triad of this new serine-type protease, wild-type and four mutant AAREs, in which each candidate residue of the catalytic triad deduced from sequence alignment with other oligopeptidases was substituted by site-directed mutagenesis, were expressed in *Escherichia coli* as fusion proteins with short peptide chains at both N- and C-termini of a subunit of porcine liver enzyme. All of the recombinant AAREs were estimated to have similar conformational and quaternary structures to the native porcine liver enzyme from their CD spectra and behavior on gel-filtration, but the mutants in which Ala^{587}, Asn^{675}, or Tyr^{707} was substituted for Ser^{587}, Asp^{675}, or His^{707}, respectively, did not show detectable hydrolytic activity toward acetyl-L-methionyl L-alanine. These facts suggest that Ser^{587}, Asp^{675}, and His^{707} are essential residues for the AARE activity and comprise the catalytic triad of the enzyme in this order. Thus, AARE has been shown to have a protease-like domain in its C-terminal region, as do other proteins classified as members of the oligopeptidase family.

Key words: acylamino acid-releasing enzyme, catalytic triad, oligopeptidase, serine protease, site-directed mutagenesis.

Amino-terminal acylation is one of the most common modifications of proteins and bioactive peptides, being widely observed in eukaryotic proteins (*1, 2*). N^{α}-Acetylation is a common event in mammalian cells, in which 60-80% of intracellular proteins are reported to be acetylated (*3*). The physiological significance of this modification, however, remains unclear. One suggestion is that it plays a role in the stability of proteins with respect to the ubiqitin-degradation system (*4, 5*). Concerning N^{α}-acetylation, it has also been suggested that the activities of some bioactive peptides are controlled by acetylation at their amino termini (*6*).

Acylamino acid-releasing enzyme (AARE, also known as acylpeptide hydrolase) [EC 3.4.19.1], which catalyzes the hydrolysis of an N^{α}-acylated peptide to an acylamino acid and a peptide with a free N-terminus (*7, 8*), is considered to be a key enzyme involved in sequential deacetylation of such N-terminally acetylated proteins. Removal of N-terminal acetyl groups of proteins is estimated to be processed as follows: (i) N-terminally acetylated proteins are degraded to small peptides by endo-type protease(s); (ii) the resulting small N-terminally acetylated peptides are then hydrolyzed by an AARE into peptides having free N-termini and N-acetylamino acids; and (iii) the N-acetylamino acids are further hydrolyzed by aminoacylase [EC 3.5.1.14] into amino acids and acetic acid.

Porcine liver AARE is a tetrameric protein of about 320 kDa with identical subunits consisting of 732 amino acid residues (*9*) and a member of the serine proteases, since it is stoichiometrically inactivated by diisopropyl fluorophosphate (*10*). Based on a sequence homology search, AARE has been recently classified as a member of the new serine-type proteases, termed the "oligopeptidase family," which have no sequence homology with well-known serine proteases such as chymotrypsin or subtilisin or with a large number of lipases and esterases (*11*) except for a consensus motif, Gly-X-Ser-X-Gly, comprising the active-site serine residue (*12*). Such proteases include porcine brain and *Flavobacterium meningosepticum* prolyl endopeptidases (PREP) (*13, 14*), *Escherichia coli* protease II (*15*), and rat and mouse liver dipeptidyl peptidases IV (DPP IV) (*16, 17*). From sequence alignment, these proteases have been found to share a strong resemblance in their C-terminal regions of 250 amino acid residues (*11, 18*), and these regions are presumed to relate to the expression of protease activities. Essential residues comprising a catalytic triad of

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Abbreviations: AARE, acylamino acid-releasing enzyme; Ac-Met-MCA, acetyl-L-methionine 4-methyl-coumaryl-7-amide; AMC, 7-amino-4-methylcoumarin; DPP IV, dipeptidylpeptidase IV; IPTG, isopropyl-1-thio-β-D-galactoside; LC/MS, liquid chromatography/mass spectrometry; MPE buffer, 1 mM 2-mercaptoethanol, 1 mM EDTA, and 5 mM sodium phosphate buffer (pH 7.2); PREP, prolyl endopeptidase; PVDF, polyvinylidene difluoride.

Ser-Asp-His, in this order, have been identified in this region of mouse DPP IV, and these residues are highly conserved in the other proteases (*19*) classified as members of the "oligopeptidase family." In case of AARE, Ser^{587} and His^{707} have been shown to be catalytic residues by chemical modification studies of human enzyme (*20*), and the participation of Ser^{587} in the activity has been confirmed by chemical modification of the porcine enzyme (*10*). However, the acidic residue of the triad in porcine liver AARE remains unclear, although Asp^{562} and Asp^{675} are suggested to be candidates from sequence alignment with the other oligopeptidases.

As one approach for elucidation of the enzymatic mechanism of AARE, we have tried to determine the catalytic triad residues by using site-directed mutagenesis of porcine liver AARE. This paper describes the construction of an expression system for production of porcine liver AARE in *E. coli*, and the mutational analyses to provide experimental evidence for the putative catalytic residues in this enzyme.

MATERIALS AND METHODS

Materials—Plasmid pET23a, His-Bind resin, anti-T7-Tag monoclonal antibody, and *E. coli* BL21(DE3) were obtained from Novagen (Madison, USA). Plasmid pUC18, PCR *in vitro* mutagenesis kit, and *E. coli* HB101 were obtained from Takara Shuzo (Kyoto). PVDF membrane (Immobilon-P) for Western blotting was the product of Millipore (Milford, USA). Goat anti-mouse IgG conjugated to horseradish peroxidase was purchased from Organon Teknika (West Chester, USA). The sources of other specific chemicals and reagents are shown in the text in each case.

Construction of Porcine Liver AARE Expression Plasmid—Two cDNA fragments, λ_{AARE} 419 and λ_{AARE} 521 described by Mitta *et al.* (*9*), which encode the C- and N-terminal parts of AARE subunit from porcine liver, were connected at an overlapping *Sac*I site and cloned into an *E. coli* expression plasmid pET23a downstream of the T7 promoter (*21, 22*) as follows. The cDNA fragment λ_{AARE} 521 was digested at the *Nco*I site including the ATG initiation codon and an *Eco*RI site. The released fragment was blunt-ended with Klenow and inserted into the blunt-ended *Bam*HI site of pET23a as shown in Fig. 1B, resulting in pEA101N. The plasmid pEA101N was then digested at *Sac*I and *Hin*cII sites on the vector and ligated with the *Sac*I-*Eco*RI fragment obtained from the cDNA fragment λ_{AARE} 419 to make plasmid pEA301T7. Finally, the *Spe*I-*Pvu*II fragment of pEA301T7 coding for the C-terminal region of AARE was placed back into pEA301T7 at the *Spe*I-*Not*I site as shown in Fig. 1C, resulting in plasmid pEA301T7-His (Fig. 1A). This plasmid was basically used as an expression plasmid for recombinant AAREs.

Site-Directed Mutagenesis of Recombinant AAREs—Plasmid pEA301T7-His was digested with *Acc*III and *Sca*I, and a 650-bp fragment encoding the C-terminal region of the AARE was isolated (see Fig. 1). The fragment was blunt-ended and subcloned into the *Hin*cII site of pUC18 to make plasmid pUAMUT. Mutations were introduced into the AARE gene in pUAMUT using the PCR *in vitro* mutagenesis kit according to the method described by Ito *et al.* (*23*). Synthetic oligonucleotides were designed to allow the desired single amino acid changes and restriction sites for screening (Table I). Mutations were verified by DNA sequencing and cloned back into the *Spe*I-*Xho*I site of pEA301T7-His.

Growth and Purification of Recombinant AAREs—*E. coli* BL21(DE3) harboring the appropriate expression plasmid was grown at 37°C to mid-log phase in 2×YT broth containing 100 μg/ml ampicillin. Then the culture was transferred to a shaking water bath at 15°C and further incubated for 15 h. All subsequent purification procedures were carried out at 4°C. Cells were harvested and resuspended in 30 ml of the binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl pH 8.0). The suspension was sonicated and centrifuged for 30 min at 16,000×*g*. The supernatant was loaded onto a Ni^{2+} chelation column (His-Bind Resin, φ 1.6×5 cm) which was charged with Ni^{2+} ions and equilibrated with the binding buffer. The column was washed with 100 ml of the binding buffer and with 60 ml of the washing buffer (60 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl pH 8.0), and eluted with a linear gradient of the concentration of imidazole from 60 to 200 mM in 20 mM Tris-HCl (pH 8.0) containing 0.5 M of NaCl. An aliquot of each fraction was monitored by SDS-PAGE and Western blotting, and AARE-containing fractions were pooled and dialyzed against the MPE buffer (1 mM 2-mercaptoethanol, 1 mM EDTA, and 5 mM sodium phosphate buffer pH 7.2). The dialysate was applied to an Econopack Mono Q cartridge column (Bio-Rad, USA) equilibrated with the MPE buffer and eluted with a linear gradient of the concentration of NaCl from 0 to 0.4 M. After monitoring each fraction by SDS-PAGE, AARE-containing fractions were saved and concentrated using a Centricon-10 device (Amicon, USA). The proteins were further purified by gel-filtration on a Superose 6 column (φ 3.2×300 mm) equilibrated with the MPE buffer containing 100 mM NaCl using the LC SMART system (Pharmacia, Sweden), and finally dialyzed against the MPE buffer.

Enzyme Assays—AARE activity was routinely measured using acetyl-L-methionine 4-methyl-coumaryl-7-amide (Ac-Met-MCA) as a substrate. An appropriate amount of the enzyme solution dissolved in the MPE buffer (usually 100 μl) was incubated at 37°C with 20 μM Ac-Met-MCA in 200 mM sodium phosphate buffer (pH 7.2) containing 0.4% DMF in a total volume of 990 μl. The reaction was terminated by adding 10 μl of 10% SDS, and the amount of 7-amino-4-methylcoumarin (AMC) released from the sub-

TABLE I. **Oligonucleotides used for plasmid construction.**

Nucleotide sequence (5′≫3′)[a]	Mutation	Diagnostic	Plasmid
GTGAAGAACGTCCAGTTTGC	Asp^{562}≫Asn^{562}	destroys *Aat*II	pEAMD562N
GGGTGGTGCACATGGTGG	Ser^{587}≫Ala^{587}	destroys *Nco*I	pEAMS587A
GATGCTTGGCCAGGAGAACAGGC	Asp^{675}≫Asn^{675}	creates *Bal*I	pEAMD675N
CCCAAAAGCACATATGCACTATC	His^{707}≫Tyr^{707}	creates *Nde*I	pEAMH707Y

[a]Mutated nucleotides are underlined.

strate was measured with a fluorescence spectrophotometer (RF-540, Shimadzu, Tokyo) with excitation at 380 nm and emission at 440 nm. One unit of the activity was defined as the amount of enzyme which released 1 μmol of AMC per min under the conditions described above. The rate of the substrate cleavage was determined by using 4 μg/ml enzyme at varied concentration of substrate (0.125, 0.25, 0.5, 1.0, or 2 mM) in 50 mM phosphate buffer (pH 7.2) for 30 min at 37°C. In these cases, acetyl-L-methionine-L-alanine (Ac-Met-Ala) was used as the substrate as described previously (*24*). Kinetic values were calculated based on Lineweaver-Burk plots.

Protein concentration was determined by the method of Bradford (*25*) with bovine serum albumin as the standard.

Electrophoresis and Immunoblotting—SDS-PAGE (7.5%) was carried out according to the method of Laemmli (*26*). Proteins were detected by staining with Coomassie Brilliant Blue. For immunoblotting, proteins on the unstained gels were electroblotted onto PVDF membranes, and the recombinant proteins were probed by using anti-T7-Tag monoclonal antibody followed by goat anti-mouse IgG conjugated to horseradish peroxidase, and detected by the use of ECL Western blotting system (Amersham, UK).

Protein Analysis—Amino acid sequences of recombinant AAREs were confirmed by LC/MS analysis. The protein samples (500 pmol) were digested with *Achromobacter* protease I in 20 mM Tris-HCl buffer (pH 9.0) containing 2 M urea at 37°C for 6 h at a molar enzyme/substrate ratio of 1/400. Aliquots of each digest (50 pmol) were loaded onto a packed reverse-phased column (0.32×150 mm, Poros II R/H, Perseptive) connected to the capillary HPLC system (model 625, Waters) and separated with linear gradient of the concentration of acetonitrile in 0.05% TFA from 0 to 80% for 40 min at a flow rate of 0.2 μl/min. The column eluent was introduced into an ionspray probe of the triple quadrupole mass spectrometer (API-III, Sciex). The total ion chromatograms were recorded in the single quadrupole mode.

Structural Analyses of AAREs—To estimate the molecular weight of the recombinant AAREs, purified AAREs were gel-filtrated on a 3.2×300 mm column of Superose 6 in the MPE buffer containing 100 mM NaCl with a flow rate of 40 μl/min at 15°C using thyroglobulin (669 kDa), ferritin (440 kDa), and catalase (232 kDa) as the standard proteins for the calibration of molecular weight. CD spectra were measured from 260 to 195 nm on a spectropolarimeter (model J720, Japan Spectroscopic) at 20°C using a 2 mm path length cuvette. Protein samples were dissolved at a concentration of 0.2 mg/ml in 5 mM sodium phosphate buffer (pH 7.2) containing 0.5 mM EDTA.

RESULTS

Expression of Wild-Type Recombinant Porcine AARE in E. coli—To express the subunit polypeptide of porcine liver AARE in *E. coli*, the plasmid pEA301T7-His, in which the AARE cDNA was cloned under the control of T7 RNA polymerase promoter (*27*), was constructed (Fig. 1). By use of this plasmid, the AARE subunit was expressed as a fusion protein of 756 amino acid residues containing 14 amino acid residues, MASMTGGQQMGRGS (T7-Tag), at the N-terminus and 10 amino acid residues, AALEHHHHHH (His-Tag), at the C-terminus, and the C-terminal Ser^{732} of the AARE subunit was substituted by an arginine residue. The first 11 amino acid sequence of T7-Tag is the same as the T7 gene *10* protein, which is the major capsid protein of phage T7 (*22*), and is useful as a tag sequence for detection of the recombinant proteins by using an anti-T7-Tag monoclonal antibody. The His-Tag contains a histidine hexamer and is effective for purification of the recombinant proteins by metal chelation chromatography (*28-30*).

E. coli BL21(DE3), which has an IPTG-inducible T7 RNA polymerase gene on its chromosome DNA, was transformed with pEA301T7-His, and the growth conditions for expression of the recombinant AARE were examined. The transformed cells were grown in 2×YT medium at 37°C to mid-log phase, then expression of the enzyme was induced by the addition of IPTG at a final concentration of 1 mM, and incubation was continued at 37°C for 16 h. Overproduction of the recombinant AARE was observed on SDS-PAGE and Western blotting of total

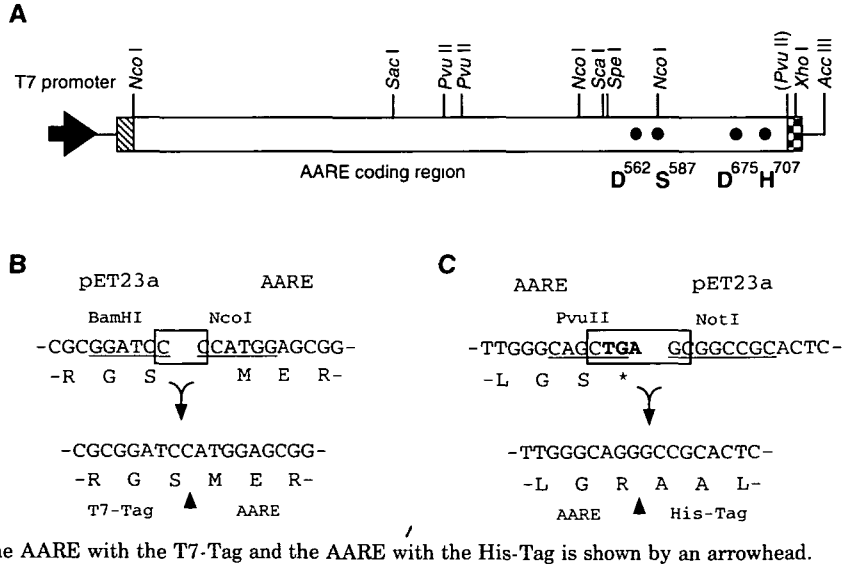

Fig. 1. **Construction of plasmids used for expression of wild-type and mutant AAREs.** The wild-type AARE expression vector, pEA-301T7-His, contains an open reading frame of 756 residues consisting of T7-Tag, MASMTGG-QQMGRGS, whole porcine liver AARE subunit, and His-Tag, AALEHHHHHH, placed under control of the T7 promoter. The C-terminal Ser^{732} of the AARE subunit was substituted by an arginine residue. (A) Restriction enzyme map of pEA301T7-His. The region from the T7 promoter to *Acc*III site on the vector is shown. The slashed, open, and hatched bars represent the T7-Tag, the AARE, and the His-Tag coding region, respectively. The positions of the residues which were substituted by site-directed mutagenesis are indicated by dots. (B and C) The fusion sites at N- and C-termini of the AARE subunit. The restriction enzyme sites on the AARE gene and pET23a are underlined. The termination codon of AARE gene is shown in bold letters. The boxes denote the bases that were cleaved prior to ligation. Each position combining the AARE with the T7-Tag and the AARE with the His-Tag is shown by an arrowhead.

cellular protein solubilized with SDS. However, very low AARE activity was detected in the supernatant of the sonicated cell extract. SDS-PAGE and Western blotting of the cell extract revealed the presence of only a faint amount of the recombinant AARE in the supernatant, suggesting that most of the recombinant protein was present in insoluble form, such as in inclusion bodies. To avoid the formation of insoluble proteins, culture conditions were further examined with regard to the effect of IPTG induction and cultivation temperature. At mid-log phase of the culture at 37°C, the culture was transferred to a shaking water bath at 30, 25, 20, or 15°C with or without addition of IPTG, and AARE activities of the cell extracts were measured. The addition of IPTG was found to decrease the AARE activity in the cell extract at each temperature, and the highest activity was recovered from the culture incubated at 15°C regardless of the addition of IPTG. Therefore, the recombinant AARE was prepared by culturing cells to mid-log phase at 37°C, then shifting the culture to 15°C without adding IPTG.

The recombinant AARE obtained as described above was purified to homogeneity by three steps of column chromatography: chelation chromatography, anion exchange chromatography, and gel filtration. By these procedures, about 1 mg of the wild-type recombinant AARE was obtained from one liter culture in a yield of 30–35%.

LC/MS analysis of *Achromobacter* protease I-digests of the wild-type recombinant AARE and native enzyme from porcine liver revealed that they were identical except for the retention times of the N- and C-terminal peptides, which had respectively 14 and 10 additional amino acid residues in the recombinant enzyme (Fig. 2). The observed mass numbers of peptides obtained from the two enzymes also agreed within acceptable limits of error with the theoretical mass numbers calculated from the amino acid sequences (Table II), except for the peptides AP 2, 3, 5, 6, 9, 10, 13, 14, 24, and 25, which were too small to be retained on the HPLC column. It was thus confirmed that the primary structure of the wild-type recombinant AARE is the same as the designed structure by the gene manipulation.

The molecular weight of the recombinant AARE subunit estimated by SDS-PAGE agreed with the calculated value (83,910) and was slightly larger than the value of the native AARE subunit, as expected. Gel filtration showed that the recombinant AARE associated as a tetramer, like the porcine liver native AARE, and had an apparent molecular mass of 340 kDa. From the CD spectra, it was estimated by the method of Chang *et al.* (*31*) that the recombinant AARE had a smaller α-helical content by 4.6% and a greater β-turn content by 4.4% than the native AARE from porcine liver, but their overall conformational structures were thought to be almost identical (Fig. 3). No significant differences were observed in their kinetic values (Table III).

Site-Directed Mutagenesis of Active Site Residues of the Recombinant AARE—Sequence alignment of new serine-type proteases belonging to the oligopeptidase family (*11, 18*) suggested that Ser^{587}, Asp^{675}, His^{707}, and Asp^{562} are candidates for residues comprising the catalytic triad in porcine liver AARE. To evaluate directly the roles of these residues, AARE mutants in which Ser^{587}, Asp^{675}, His^{707}, or Asp^{562} was changed to Ala^{587}, Asn^{675}, Tyr^{707}, or Asn^{562}, respectively, were prepared and their enzymatic properties were examined. The plasmids expressing these AARE mutants were constructed by introducing mutations in the AARE cDNA on plasmid pEA301T7-His as described in "MATERIALS AND METHODS." *E. coli* BL21(DE3) cells were transformed with the plasmids, and the recombinant AARE mutants were prepared by the same procedures as used for the recombinant wild-type AARE. The behaviors of the mutant enzymes at each purification step were almost the same as those of the recombinant wild-type AARE.

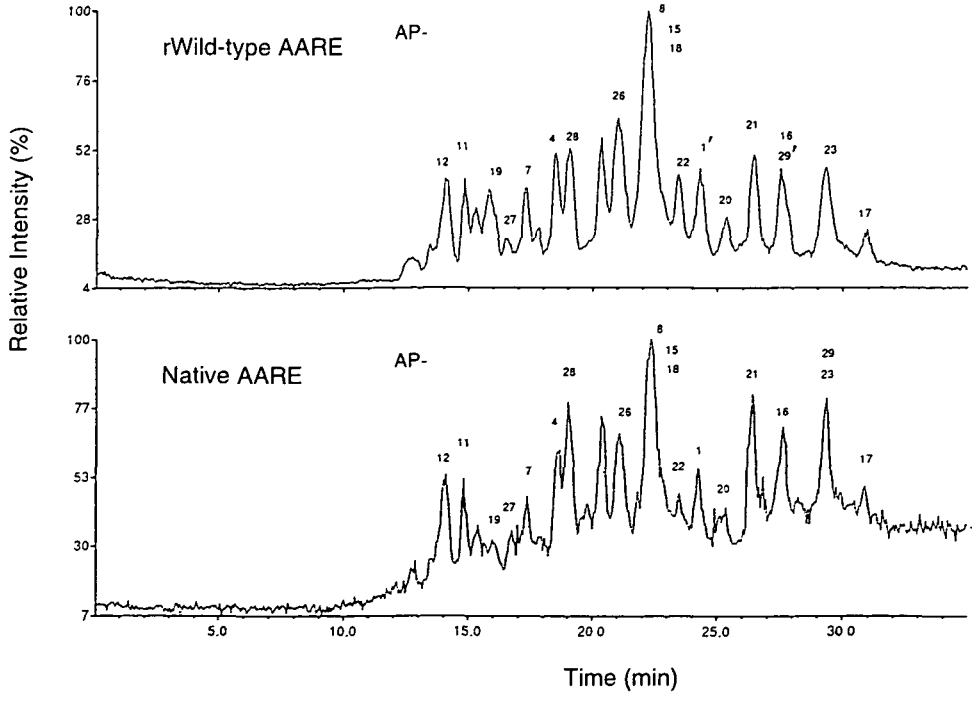

Fig. 2. **Total ion current chromatograms of *Achromobacter* protease I-digested peptides from porcine liver and recombinant AAREs.** The protein samples (500 pmol) were digested with *Achromobacter* protease I (1.25 pmol) in 20 mM Tris-HCl buffer (pH 9.0) containing 2 M urea at 37°C for 6 h. One-tenth of the resulting peptides was separated on a reverse-phased capillary column and consecutively introduced into a mass spectrometer as described in the text. The peptides (AP-), whose mass numbers were observed to be identical with theoretical numbers of *Achromobacter* protease I-digested peptides, are denoted.

TABLE II. **Peptides obtained from *Achromobacter* protease I-digest of each AARE and their molecular masses.**

Peptide[a]	Calculated	Observed		Residues[b]
		Native AARE	rWild-type AARE	
AP1	11,606.1	11,606.0	—	1-103
AP1′	12,944.6	—	12,945.2	−14-103
AP2	585.8	N.D.[c]	N.D.	104-108
AP3	920.0	N.D.	N.D.	109-118
AP4	1,078.2	1,078.0	1,077.7	119-126
AP5	416.5	N.D.	N.D.	127-129
AP6	259.4	N.D.	N.D.	130-131
AP7	1,008.1	1,008.0	1,007.7	132-140
AP8	3,223.6	3,222.7	3,223.5	141-168
AP9	146.2	N.D.	N.D.	169
AP10	399.5	N.D.	N.D.	170-172
AP11	957.1	956.7	956.7	173-180
AP12	1,608.8	1,609.0	1,608.0	181-195
AP13	146.2	N.D.	N.D.	196
AP14	670.8	N.D.	N.D.	197-202
AP15	2,065.3	2,065.0	2,065.2	203-219
AP16	7,856.0	7,855.6	7,855.7	220-291
AP17	13,236.1	13,236.2	13,237.0	292-411
AP18	2,557.1	2,557.2	2,556.5	412-434
AP19	885.1	884.5	884.7	435-443
AP20	6,131.8	6,132.0	6,132.0	444-497
AP21	3,301.1	3,300.3	3,301.5	498-527
AP22	3,578.1	3,577.7	3,577.7	528-561
AP23	10,117.5	10,118.1	10,118.1	562-654
AP24	443.6	N.D.	N.D.	655-658
AP25	704.8	N.D.	N.D.	659-664
AP26	2,013.4	2,013.0	2,013.0	665-681
AP27	1,286.5	1,286.0	1,286.0	682-691
AP28	1,538.9	1,539.0	1,539.0	692-705
AP29	3,063.4	3,063.0	—	706-732
AP29′	4,339.8	—	4,340.1	706-742

[a]Peptides predicted to be produced by *Achromobacter* protease I-digestion of porcine liver AARE are numbered sequentially from the N-terminal peptide. The N- and C-terminal peptides of the recombinant wild-type AARE are shown by dashed numbers (AP1′, AP29′). [b]Amino acid residues are numbered from the N-terminal acetylmethionine residue of porcine liver AARE. Negative numbers are used for the N-terminal extension of the recombinant enzyme. [c]ND, not detected in these experimental conditions.

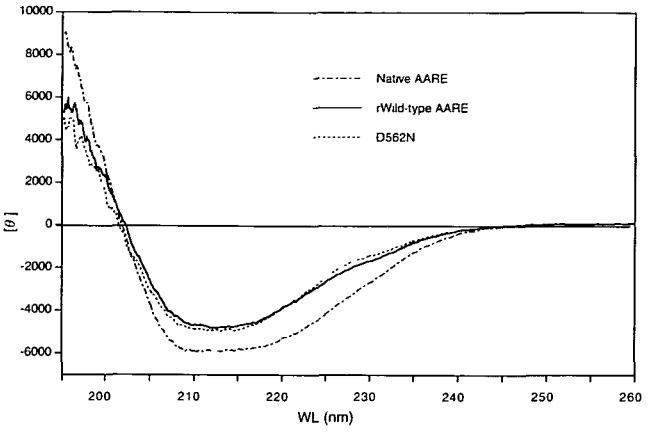

Fig. 3. **Circular dichroism spectra of native porcine liver and recombinant AAREs.** Protein samples were dissolved at a concentration of 0.2 mg/ml in 5 mM sodium phosphate buffer (pH 7.2) containing 0.5 mM EDTA and the CD spectra from 260 to 195 nm of the sample were measured. Only the CD spectra of the native, the recombinant wild-type, and the mutants D562N are shown. The CD spectra of the mutants S587A, D675N, and H707Y were identical with that of the recombinant wild-type AARE.

TABLE III. **Kinetic values of porcine liver AARE and its recombinant enzymes.**[a]

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/mM^{-1})
Native AARE	0.49	33	67
rWild-type rAARE	0.56	29	51
D562N mutant	0.68	13.4	20
S587A mutant	NA[b]	NA	—
D675N mutant	NA	NA	—
H707Y mutant	NA	NA	—

[a]Kinetic values were determined using Ac-Met-Ala as substrate. [b]NA, no detectable activity.

The primary structures of the mutants were analyzed using LC/MS. The chromatograms of *Achromobacter* protease I-digests of the mutants and the theoretical and observed mass numbers of peptides, each of which includes a substituted amino acid residue, are shown in Fig. 4. Each of the observed mass numbers of these peptides agrees within acceptable limits of error with the theoretical mass numbers, indicating that the mutants have primary structures that are exactly the same as the designed structures by site-directed mutagenesis.

To confirm the structural integrity of these mutant enzymes, their molecular weights were analyzed. SDS-PAGE analysis showed that the molecular weight of each mutant AARE subunit was approximately 84 kDa, which was the same as that of the recombinant wild-type AARE. On Superose 6 gel-filtration column (ϕ 3.2×300 mm), all mutant AAREs were eluted at the same position as the recombinant wild-type AARE, corresponding to about 340 kDa, indicating that the mutants also existed as tetramer. The CD spectra of the mutants S587A, D675N, and H707Y overlapped that of the recombinant wild-type AARE, whereas the mutant D562N showed a slightly different pattern (Fig. 3). The calculated α-helical content of this mutant was 14.8%, which was smaller than that of the recombinant wild-type AARE by 3.1%. These results suggest that only the substitution of Asn^{562} for Asp^{562} affected the conformation of surrounding polypeptide chain.

The enzymatic activities of the mutant AAREs were then assayed by use of Ac-Met-Ala as substrate (Table III). None of the mutants S587A, D675N, and H707Y showed detectable activity, indicating that Ser^{587}, Asp^{675}, and His^{707} participate in the formation of the catalytic triad of porcine liver AARE. On the other hand, the K_m value of the mutant D562N showed no significant difference from that of the recombinant wild-type AARE, but its k_{cat} value was half of that of the recombinant wild-type AARE.

DISCUSSION

To study the structure-function relationship of AARE, porcine liver AARE was expressed in *E. coli* as a fusion polypeptide with an N-terminal leader sequence, since no production of the polypeptide was observed directly from its start codon. At 37°C, the AARE was expressed as an insoluble complex, such as an inclusion body, and limitation of the inducer concentration resulted in increase of the active form of the enzyme. However, AARE activity detected in the extract was low, and several sequential bands migrating faster than the expected size of the product

were observed on Western blotting. This suggests that the recombinant AARE is degraded in the *E. coli* cells. Therefore, a new expression system was required in order to obtain soluble recombinant AARE in a large amount. This problem was solved by lowering the temperature of the culture from 37 to 15°C at the mid-log phase of cell growth. This suppressed the degradation and increased the amount and the enzymatic activity of the recombinant AARE. This method was especially effective for production of the mutant D562N, which was rather unstable *in vivo*.

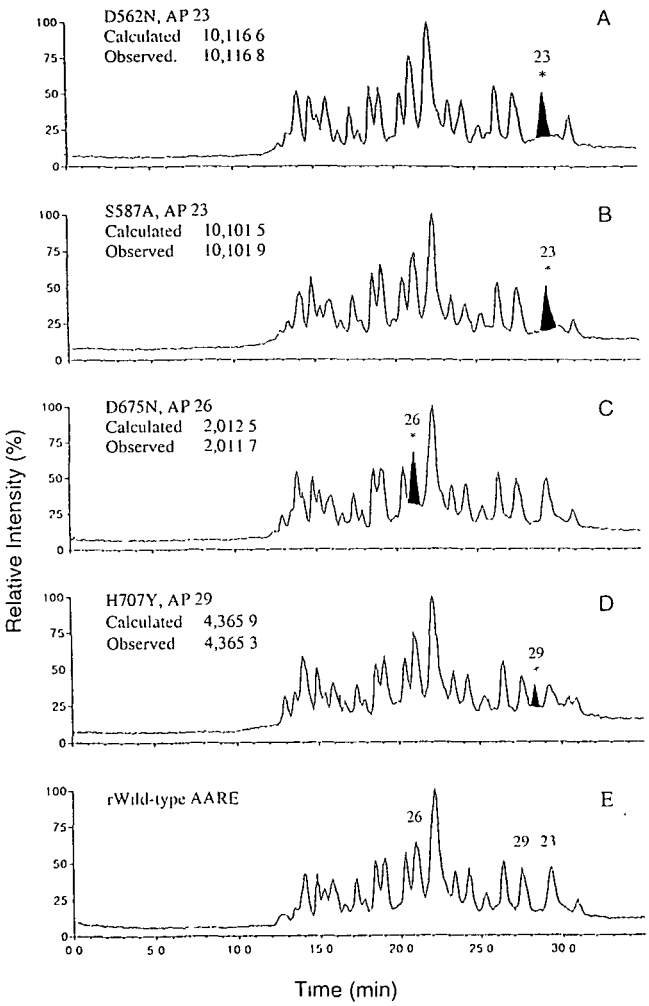

Fig. 4. **Total ion current chromatograms for *Achromobacter* protease I-digested peptides from the recombinant wild-type and mutant AAREs.** LC/MS analyses were performed as described in the legend to Fig. 2. Each peak containing a peptide with a substituted residue by site-directed mutagenesis is shown by an asterisk (*). Theoretical and observed mass numbers of the above peptides are shown in the figure.

The recombinant AARE subunit was thus expressed as a fusion protein with a leader peptide of 14 amino acid residues, T7-Tag, at its N-terminus and a peptide tail of 10 amino acid residues, His-Tag, at its C-terminus. The structural properties of the recombinant wild-type AARE were compared with those of the native AARE purified from porcine liver. CD spectra of the recombinant AARE indicated that it had a lower α-helical content than the native AARE. This difference may be caused by the presence of the extra N- and C-terminal sequences of the recombinant enzyme extended by T7-Tag and His-Tag. Six histidine residues included in the His-Tag are likely to be responsible for the ellipticity due to its adsorption band around 211.5 nm. The results of both SDS-PAGE and gel filtration analyses indicate that the recombinant enzyme has almost the same quaternary structures as the native enzyme. This was supported by kinetic studies demonstrating that the recombinant wild-type AARE is not significantly different from the native AARE in its enzymatic properties, despite the presence of the additional N- and C-terminal sequences. These results demonstrate that this expression system for the AARE is suitable for use in mutational analyses of other enzymes.

The mutants in which Ala^{587}, Asn^{675}, or Tyr^{707} was substituted for Ser^{587}, Asp^{675}, or His^{707}, respectively, had no detectable hydrolytic activity toward Ac-Met-Ala, although their CD spectra and behaviors on gel filtration indicated that they have similar conformation to the recombinant wild-type AARE. These results strongly suggest that Ser^{587}, Asp^{675}, and His^{707} are essential residues for expression of the activity by comprising the catalytic triad in porcine liver AARE.

This finding that Ser^{587} is an essential residue for the AARE activity agrees with the result of chemical modification study with [^{3}H]diisopropyl fluorophosphate (*10*). It also agrees with the results of chemical modification studies of human erythrocyte AARE (*20*). The involvement of Ser^{587} in the catalytic triad is further supported by both chemical modification studies (*13, 16, 32*) and site-directed mutagenesis studies (*16, 19*) of other proteases classified as members of the oligopeptidase family.

The result that His^{707} is also an essential residue for the

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                                        *
AARE    536 NYRGSTGFGQDSILSLPGNVGHQDVKDVQFAVEQVLQEEHFDAGRVALMGGS587HGGFLSCHLICQYPETYSACVV 610
DPP IV  573 DGRGSGYQGDKIMHAINRRLGTLEVEDQIEAARQFVKMGFVDSKRVAIWGWS624YGGYVTSMVLGSGSGVFK-CGI 645
PREP    503 NIRGGGEYGETWHKGGILANKQNCFDDFQCAAEYLIKEGYTSPKRLTINGGS554NGGLLVATCANQRPDLFG-CVI 575

                    *                                                   *
AARE    654 KSPIKYAPQVKTPLLLMLGQED675RRVPFKQGMEYYRVLKARNV-----PVRLLLY--PKST707LSEVEVESDSFM 720
DPP IV  683 MSRAEHFKQVE--YLLIHGTADD702NVHFQQSAQISKVLVDAGVD-----FQAMWY--TDEDH736GIASSTAHQHIYS 747
PREP    621 KLPEADDIQYPSMLLLTADHDD642RVVPL-HSLKFIATLQYIVGRSRKQNNPLLIHVDTKAGH680GAGKPTAKVIEEV 693
```

Fig. 5. **Alignment of the amino acid sequences around the catalytic triad residues in porcine liver AARE, mouse liver DPP IV, and porcine brain PREP.** Asterisks (*) indicate the catalytic triad residues. Conserved residues between more than two enzymes are shown in white on black.

AARE activity agrees with the results of chemical modification of the human enzyme using acetylleucine chloromethylketone (*20*). The active histidine residues of other oligopeptidase have been identified as His^{734} and His^{680} in the mouse DPP IV (*19*) and the porcine brain PREP (*33*), respectively. These residues correspond to His^{707} in the AARE from its sequence alignment.

The remaining unidentified residue comprising the catalytic triad in AARE is presumably aspartic acid. Asp^{702} in the mouse DPP VI has been shown to be related to the enzymatic activity by site-directed mutagenesis. This residue corresponds to Asp^{675} in the porcine liver AARE. Therefore, Asp^{675} is most likely to be a member of the catalytic triad in the porcine liver AARE. To clarify the contribution of Asp^{675} to the enzymatic activity, mutants in which an asparagine residue was substituted for Asp^{675} (mutant D675N) or Asp^{562} (mutant D562N), which is another candidate residue as a member of the catalytic triad, were prepared, and their activity was examined. The substitution of asparagine for Asp^{562} or Asp^{675} had different affects on the AARE activity.

Mutant D562N retained the activity toward Ac-Met-Ala substrate, although the k_{cat} value was slightly lower than that of the recombinant wild-type AARE. This lower activity is thought to reflect the instability of this mutant protein, which is evident in its less efficient expression than the other mutant proteins in cells cultivated at 37°C. The CD spectrum of the mutant D562N indicated a slightly lower α-helical content than other mutants. The secondary structure of this mutant protein predicted by Chou and Fasman (*34*) suggests that an α-helix structure involving the Asp^{562} residue in the recombinant wild-type AARE is changed to a β-sheet structure in the mutant protein. The importance of an α-helix structure involving about 100 residues around Asp^{562} is also suggested by the fact that this region is completely conserved among porcine, rat, and human AAREs (*35*). The loss of stability by the substitution of this positional aspartic acid residue is also observed in the mutant enzyme of the mouse DPP VI (*19*), which has an alanine residue in place of the aspartic acid residue. All of these observations indicate that an α-helix structure involving the Asp^{562} residue plays an important role in the stabilities of proteases belonging to the oligopeptidase family, including AARE.

On the contrary, the mutant D675N completely lost the activity toward Ac-Met-Ala. This fact strongly suggests that Asp^{675} is a member of the catalytic triad of AARE. This result agrees with the results obtained from the mutational analysis of the mouse DPP VI (*19*).

In conclusion, our findings indicate that Ser^{587}, Asp^{675}, and His^{707} of porcine liver AARE are essential residues for its activity and likely to comprise the catalytic triad, Ser-Asp-His, in this order. This order differs from those observed in chymotrypsin- and subtilisin-type serine proteases, whose catalytic triads are assigned as His-Asp-Ser and Asp-His-Ser, respectively. Figure 5 shows amino acid sequences around Ser^{587}, Asp^{675}, and His^{707} of porcine liver AARE with the aligned sequences of mouse liver DPP VI (*17*) and porcine brain PREP (*13*).

Each enzyme classified as an oligopeptidase consists of a polypeptide chain of more than 70 kDa or its associates and has catalytic residues in its C-terminal region. Enzymes of this family, including AARE, are thus presumably multi-domain proteins having a protease-like domain in their C-terminal region and another functional domain in their N-terminal region. It has been reported that nicking of PREP with trypsin, which generates one fragment with a molecular weight of 26 kDa in the N-terminal region and another of 51 kDa in the C-terminal region, caused an increase in its enzymatic activity, although it is not clear at this stage whether the N-terminal domain of PREP really acts as a regulatory domain (*36*). Nicking of bovine lens AARE with trypsin at a site corresponding to the Lys^{195}-Lys^{196} bond in porcine liver AARE also generated two fragments, one with a molecular weight of 22 kDa in the N-terminal region and another of 55 kDa in the C-terminal region (*37*), although the nicked AARE did not show any greater activity than the native protein in this case. Such similarity in domain structures between these enzymes as observed above is of interest in considering the physiological functions of these proteases. The functions of AARE other than its acylamino acid-releasing activity are still not known. To clarify the function-structure relationship of AARE, we must do more research to uncover the function of the N-terminal region, the domain structure, and the significance of its tetrameric structure.

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