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Review

Cathepsin A/protective protein: an unusual lysosomal multifunctional protein

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Abstract. Cathepsin A/protective protein [3.4.16.5], carboxypeptidase A, is a lysosomal serine protease with structural homology to yeast (*Saccharomyces cerevisiae*) carboxypeptidase Y. Cathepsin A is a member of the α/β hydrolase fold family and has been suggested to share a common ancestral relationship with other α/β hydrolase fold enzymes, such as cholinesterases. Several lines of evidence indicate that cathepsin A is a multicatalytic enzyme with deamidase and esterase in addition to carboxypeptidase activities. Cathepsin A was recently identified in human platelets as deamidase. In vitro, it hydrolyzes a variety of bioactive peptide hormones including tachykinins, suggesting that extralysosomal cathepsin A plays a role in regulation of bioactive peptide functions. Recent reports emphasize the lysosomal protective function of cathepsin A rather than its protease function. The protective function of cathepsin A is distinct from its catalytic function. Human lysosomal β-galactosidase and neuraminidase exist as a high molecular weight enzyme complex, in which there is a 54-kDa glycoprotein termed 'lysosomal protective protein'. Based on cell culture studies, protective protein was found to protect both β-galactosidase and neuraminidase from intralysosomal proteolysis by forming a multienzyme complex and was shown to be deficient in patients with galactosialidosis, a combined deficiency of β-galactosidase and neuraminidase. Molecular cloning and gene expression studies have disclosed that protective protein is cathepsin A. The cathepsin A precursor has the potential to restore both β-galactosidase and neuraminidase activities in fibroblasts from patients with galactosialidosis. Cathepsin A knockout mice showed a phenotype similar to human galactosialidosis and the deficient phenotype found in the mutant mice was corrected by transplanting erythroid precursor cells overexpressing cathepsin A. Collectively, these findings demonstrate the significance of cathepsin A as a key molecule in the onset of galactosialidosis and also highlight the therapeutic potential of the cathepsin A precursor for patients with galactosialidosis.

Key words. Cathepsin A; lysosomal protective protein; carboxypeptidase; deamidase; esterase; neuraminidase; β-galactosidase; enzyme complex; galactosialidosis; peptide hormones; α/β hydrolase fold.

Introduction

Lysosomal hydrolases are responsible for the degradation of macromolecules to their building blocks, such as monosaccharides, fatty acids, and amino acids. More than 90% of all proteins are digested by lysosomal proteases with cathepsins, the predominant proteases. To date, over 20 cathepsins have been identified [1–7], and some of them are found outside lysosomes [7–9]. In addition to digestive functions, some cathepsins are involved in proteolytic maturation of lysosomal proteins and enzymes [10–14], bone remodeling [15], presentation of exogeneous antigen [16], regulation of T-cell cytolytic activity [5] and regulation of fertilization

[17]. Cathepsins can be classified into two groups, endopeptidases and exopeptidases; however, cathepsins B and H uniquely show both exo- and endo-protease activities [18, 19].

Cathepsin A (lysosomal carboxypeptidase A) was originally found in bovine spleen extract using a substrate for pepsin, CBZ-Glu-Tyr [20], and was termed 'carboxypeptidase A, lys' [21]. It is classified into the exopeptidase category and belongs to the family of serine proteases [22]. During the last decade, cathepsin A has been shown to have both catalytic and protective functions. Cathepsin A has three distinctive hydrolytic activities for carboxypeptidase [23–25], deamidase and esterase [25–28]. A deamidase that deamidates tachykinin peptides was recently purified from culture media of thrombin-stimulated human platelets and was subsequently identified as cathepsin A [24]. The enzyme catalyzes hydrolysis of a variety of bioactive peptide hormones each with specific substrate specificities [26, 27]. These findings suggest that extralysosomal cathepsin A may be involved in regulation of peptide hormones. Besides its enzymic functions, cathepsin A plays an important role in protection of lysosomal β-galactosidase and neuraminidase from intralysosomal proteolysis by forming a macromolecular complex [29, 30]. Furthermore, cathepsin A deficiency was found in patients with galactosialidosis [29], and gene disruption of cathepsin A in mice was shown to give rise to a pheno-

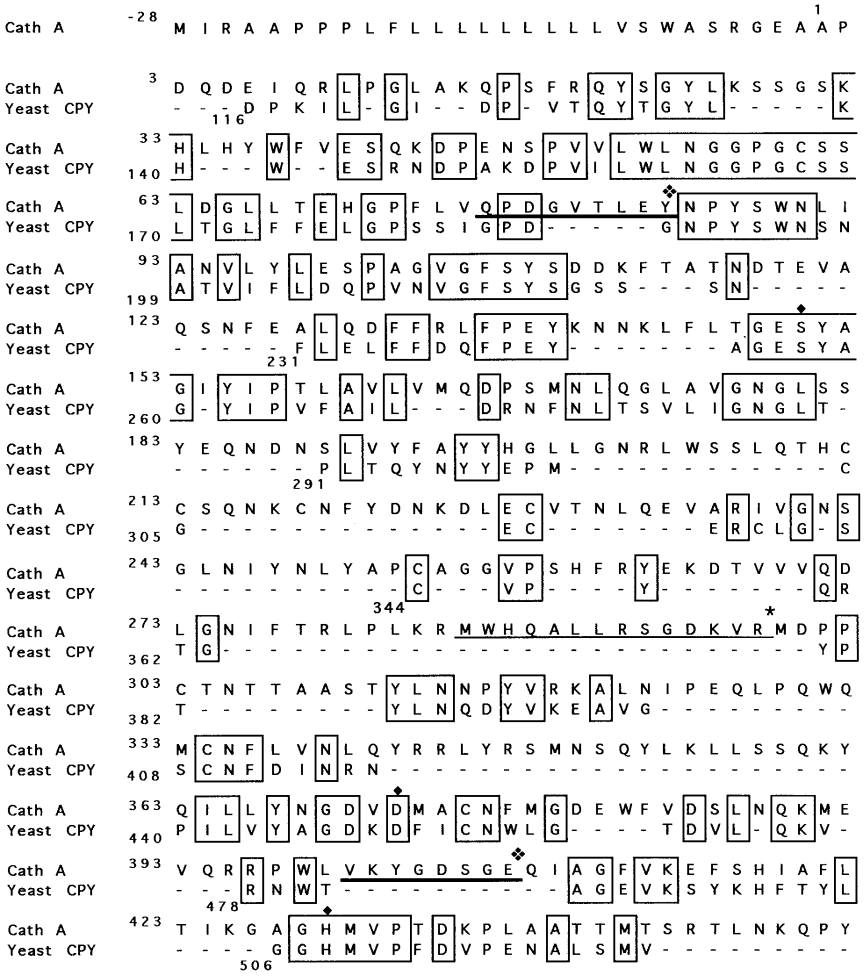

Figure 1. Amino acid sequences of human protective protein/cathepsin A and yeast carboxypeptidase Y. The amino acid sequence of human cathepsin A (Galjart et al. [32]) was aligned with that of yeast carboxypeptidase Y (Valls et al. [33]). The sequences with homology are boxed. The 32- and 20-kDa subunits locate in ^{1}Ala–^{284}Arg and ^{299}Met–^{452}Tyr, respectively. ◆ Catalytic triad, ❖ the proposed β-galactosidase-interfaces; ^{400}Val–^{407}Glu and ^{76}Gln–^{84}Tyr, ✸ excision peptide.

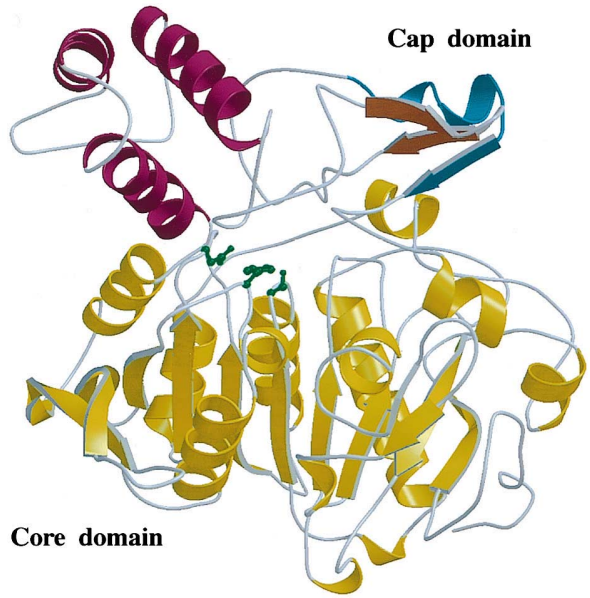

Figure 2. Three-dimensional structure of human protective protein/cathepsin A Schematic ribbon diagram of the cathepsin A monomer. The core and cap domains are shown in yellow and red, respectively. A subdomain indicated by orange is the maturation domain in which there is the excision peptide shown in light blue. The amino acids of the catalytic triad are in green. (adapted from G. Rudenko et al., 1995)

type similar to human galactosialidosis [31]. These findings demonstrate the physiological importance of cathepsin A and its multifunctional nature. The present review describes the structure and multifunctionality of cathepsin A.

Structure of cathepsin A

Deduced amino acid sequence and three-dimensional structure

The full-length complementary DNA (cDNA) for cathepsin A codes for 480 amino acids, with the first 28 residues representing a putative signal peptide as shown in figure 1 [32]. It has a structural homology to yeast and plant carboxypeptidases and the KEX-1 gene product. Human cathepsin A is composed of 32- and 20-kDa polypeptides [26, 32], both of which are linked together by disulfide bonds to form the 54-kDa cathepsin A monomer. The cDNA contains sequences coding both polypeptides. Using [^{3}H]-dipropylfluorophosphate (DFP), an inhibitor of serine protease, the catalytic site of cathepsin A was located to the 32-kDa peptide chain. The structural similarity of cathepsin A to yeast carboxypeptidase Y is also shown in figure 1.

The cathepsin A precursor is proteolytically processed to the enzymatically active mature form [34]. Using the recombinant precursor protein, a three-dimensional structure of cathepsin A was projected in relation to this activation mechanism [35, 36]. X-ray diffraction data of the crystals grown in polyethylene glycol 8000 revealed that cathepsin A monomer had dimensions of $60 \times 50 \times 70$ Å and formed a dimer in a crystal structure. The monomer is composed of two steric 'core' and 'cap' domains (fig. 2). Cathepsin A contains four disulfide bonds (^{60}Cys–^{334}Cys, ^{325}Cys–^{303}Cys, ^{212}Cys–^{226}Cys, and ^{213}Cys–^{218}Cys), of which the ^{60}Cys–^{334}Cys disulfide bond is suggested to play an important role in creating a proper conformation for interaction with substrates. The cap domain consists of a 121-amino acid sequence forming three α helixes and three-stranded β sheets in which there is a maturation site containing the 2-kDa excision peptide (^{285}Met–^{298}Arg). Excision of the 2-kDa peptide yields an enzymatically active monomer unit of cathepsin A composed of the 32- and 20-kDa polypeptides [34]. A similar linker excision in the middle of the structure can be seen in barley carboxypeptidase II [37]; however, it is not known whether this excision is required for the expression of enzyme activity. On the other hand, unlike cathepsin A and the barley enzyme, there is no maturation site in the yeast enzyme precursor, which is a significant difference in structure between cathepsin A and the yeast enzyme. Yeast carboxypeptidase Y contains an N-terminal 91-amino acid residue that must be cleaved off to convert the inactive zymogen to the active enzyme [33]. The 91-amino acid prosequence has been proposed as a 'cotranslational chaperone' necessary for a properly folded structure [38].

The α/β hydrolase fold and cathepsin A

Cathepsin A is a member of the α/β hydrolase fold enzyme family. The α/β hydrolase fold is a new concept proposed from the standpoint of protein structure evolution and is observed commonly in several functionally different hydrolytic enzymes with no sequence homology, such as acetylcholinesterase and butyrylcholinesterase [39]. Comparison of six members of the α/β hydrolase fold family in three-dimensional structure is shown in figure 3. The family has a similar core structure consisting of an α/β sheet of eight β sheets connected by α helixes, which creates a conserved catalytic triad. Although only the His residue in the catalytic triad is conserved, the nucleophil and the acid positions in the triad can be replaced with Ser, Cys, or Asp and Glu or Asp to create different catalytic activities, respectively. The catalytic triad in cathepsin A has been proposed to reside at the amino acid residues ^{150}Ser, ^{429}His, and ^{372}Asp [35]. Despite showing only 20% sequence similarity to cathepsin A, the catalytic triads of yeast and wheat carboxypeptidases preserve a topologically similar location to that of cathepsin A; both carboxypeptidases contain a ^{146}Ser/^{397}His/^{336}Asp catalytic triad [40, 41]. The cholinesterases possess peptidase,

amidase as well as esterase activities [42, 43] and have an active serine residue in a common Ser/His/Glu catalytic triad [44], implying a common ancestral relationship for yeast carboxypeptidase Y, cathepsin A and the cholinesterases.

Tissue distribution and substrate specificity of cathepsin A

Tissue distribution

Tissue distribution of cathepsin A was investigated using antibodies raised against synthetic peptides encompassing amino acid sequences in human cathepsin A [45]. Immunoblot analysis demonstrated that human cathepsin A was variably distributed in its mature form in a variety of tissues with kidney, liver and lung containing relatively higher levels than brain. In mice, higher levels of cathepsin A messenger RNA (mRNA) were observed in kidney and placenta [46]. As mentioned, cathepsin A was also shown to be present in culture media of thrombin-stimulated human platelets, suggesting an extracellular distribution [26].

Substrate specificity

Specificity on synthetic substrates. According to the amino acid preferences at the P1′ position (the Schechter and Berger nomenclature [47]), serine car

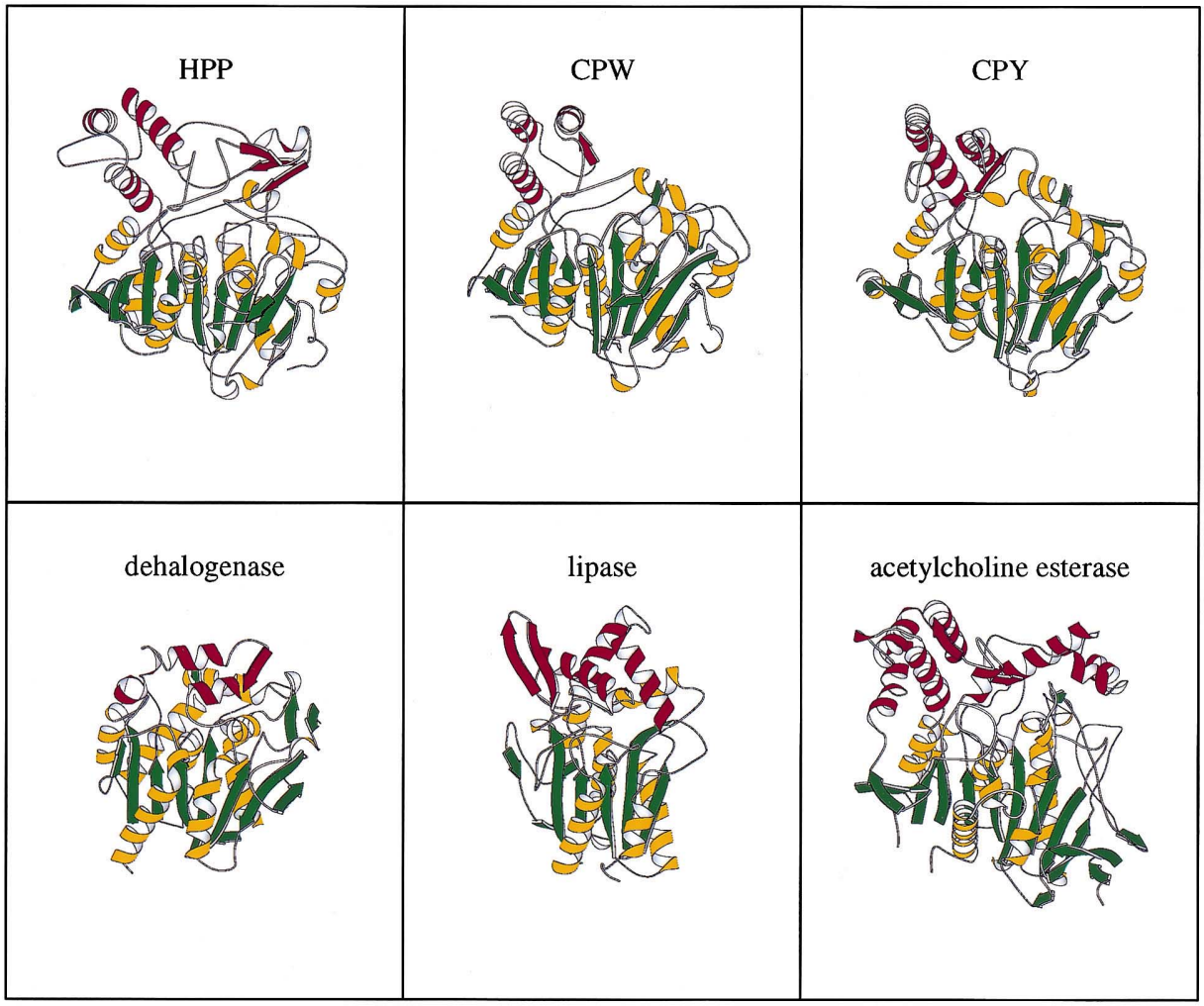

Figure 3. Comparison of six members of the hydrolase fold family in three-dimensional structure. HPP, human protective protein/cathepsin A; CPW, wheat carboxypeptidase II; CPY, yeast carboxypeptidase Y; dehalogenase from *Xanthobacter autotrophicus*; lipase from *Geotrichum candidum*; acetylcholinesterase from *Torpedo californica* [35]. The core and cap domains were in yellow and green and in red, respectively. (adapted from G. Rudenko et al., 1995)

Table 1. Comparison of carboxypeptidase activities of cathepsin As and yeast carboxypeptidase Y.

Substrate	Bovine liver	Chicken liver	Human placenta	Yeast
			(%)	
CBZ-				
Phe-Leu	100	100	100	100
Phe-Tyr	69.1	95.1	–	69.1
Phe-Ala	41.6	35.2	–	41.6
Ala-Leu	19.4	24.3	–	19.4
Phe-Gly	0.7	0.4	2.6	0.7
Ile-Gly	0.3	0.2	–	0.3
Leu-Gly	1.8	1.0	–	1.8
Ala-Glu	1.5	0.4	–	1.5
Gly-Phe	1.4	1.1	3.5	1.4
Gly-Leu	0.8	0.4	–	0.8
Glu-Phe	1.9	3.8	–	1.9
Glu-Tyr	1.4	0.8	–	1.4
Gly-Gly	0.7	0.1	3.8	0.7
Reference	52	52	52	49

Carboxypeptidase activities toward CBZ-dipeptides were expressed as a percentage of the activity toward CBZ-Phe-Leu.

boxypeptidase can be classified into two types. Carboxypeptidases with high affinity for hydrophobic amino acids and for basic amino acids at the P1′ position are classified as carboxypeptidase C and carboxypeptidase D, respectively [48]. Yeast carboxypeptidase Y belongs to the carboxypeptidase C family because it preferentially hydrolyzes peptide substrates with hydrophobic amino acids at the C-terminal [49], while wheat carboxypeptidase II has a capacity for binding to positively charged P1′ residues as well as hydrophobic amino acid residues and is classified as a carboxypeptidase D [50]. A theoretical analysis demonstrated that human cathepsin A has high affinity toward hydrophobic amino acids at the P1′ position similar to carboxypeptidase Y [51], indicating that cathepsin A can be classified as a member of the carboxypeptidase C family. As summarized in table 1, purified cathepsin As from bovine liver and chicken liver hydrolyzed dipeptide substrates with two hydrophobic amino acids in sequence better than those with one or none [52], a specificity that indicates cathepsin A is similar to yeast carboxypeptidase Y [49].

Specificity on biologically active peptides. Like many proteins, bioactive peptide hormones undergo post-translational modifications, including signal peptide cleavage, tyrosyl sulfation, phosphorylation, proteolytic processings and amidation [53]. Almost half of the known biologically active peptide hormones have C-terminal amidation that is often required for the activity [54, 55]. Trypsin-like, chymotrypsin-like and renin-like activities have been shown to be involved in prohormone processing. Of these, KEX-1 and -2 [56, 57], PAM (peptidyl-glycine-α-amidating monooxygenase) [58] and PCE (prohormone processing enzyme) [59] are the most extensively characterized.

Cathepsin A is active toward various naturally occurring bioactive peptides in vitro (table 2). The potential ability of cathepsin A to change bioactivity of peptide hormones was first described by Matsuda [60]. Subsequently, cathepsin A was demonstrated to be involved in the conversion of angiotensin I to angiotensin II by purified porcine kidney enzyme [61], suggesting that cathepsin A may be an angiotensin-activating enzyme and an important member of the renin-angiotensin system. Cathepsin A catalyzed hydrolysis of a 7-mer enkephalin, Met-enkephalin-Arg-Phe, to Met-enkephalin via formation of the Met-enkephalin-Arg intermediate [62]. Met-enkephalin-Arg-Phe has been shown to have higher analgesic activity than Met-enkephalin and to coexist with Met-enkephalin-Arg in striatum [63, 64]. These findings suggest involvement of cathepsin A in regulation of opioid peptides in brain. Interestingly, cathepsin A cleaves C-terminal amino acid from CBZ-Phe-Leu [24, 52, 65], but not from Leu-enkephalin-Phe-Leu [26], suggesting that cathepsin A may have a different mode of action on small peptides or artificial dipeptide substrates. Cathepsin A had a preference for the C-terminal Arg residue of the enkephalin peptides like carboxypeptidase B. A weak carboxypeptidase B-like activity has been demonstrated in purified rat brain cathepsin A [62], and the C-terminal Arg residue of bradykinin is also cleaved by cathepsin A [26], a specificity consistent with its effect on Met-enkephalin-Arg. Vasopressin was a poor substrate for cathepsin A compared with oxytocin, despite its 9-amino acid sequence similarity (table 2). This appears to be due to an Arg-Leu substitution at the penultimate position from the

C-terminal ends. Of the peptides examined, endothelin-1 is the best substrate for cathepsin A and is hydrolyzed about 5- to 10-fold faster than angiotensin I and substance P [27].

Deamidation and inactivation of substance P by calpains, acetylcholinesterase and butyrylcholinesterase are typical examples indicating the physiological significance of deamidase function on the regulation of small peptide hormone [44, 66]. In addition to the carboxypeptidase function, cathepsin A also catalyzes deamidation of amide-blocked peptides [26, 27]. The deamidation activity of cathepsin A prevails over carboxypeptidase activity at neutral pH, whereas the carboxypeptidase activity has an optimum pH of 5.5. Cathepsin A catalyzes the hydrolysis of amide bonds in tachykinin peptides, such as substance P and enkephalinamides, but not those in vasopressin and oxytocin (table 2). Cathepsin A cleaved off the C-terminal amidated amino acid, Gly-NH_2, from oxytocin, without hydrolyzing the amide bond. The major hydrolytic product of substance P by cathepsin A was Met-OH, indicating that deamidation occurred during hydrolysis.

Table 2. Substrate specificity of cathepsin A on bioactive peptides.

Peptide	Sequence	Relative activity (%)
Endothelin 1	Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys- Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp-OH	100
Angiotensin I	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH	9.5
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	6.1
Leu-Enkephalin	Tyr-Gly-Gly-Phe-Leu-OH	<0.5
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	3.8
Substance P(acid form)	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-OH	21.6
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2	4.0
Oxytocin (acid form)	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-OH	10.4
Vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2	<0.5
Vasopressin (acid form)	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-OH	0

▼: Sciccile bond.

Peptidase and amidase activities were measured at pH 5.5 and pH 7.0, respectively [26, 27]. All values above were expressed as a percentage of the activity toward endothelin 1 (23 μmol/min per mg protein at 0.1 mM concentration).

However, it is unclear whether deamidation occurs before or after carboxypeptidase action. All these findings support the involvement of cathepsin A in regulating peptide hormones. However, clinical symptoms associated with irregular turnover of bioactive peptides have not been investigated in the cathepsin A knockout mice [31]. Further investigation with these mutant mice will be necessary to clarify the connection between cathepsin A and the regulation of bioactive peptides.

Cathepsin A contains esterase activity toward an artificial substrate, BTEE (benzoyltyrosine ethyl ester) [25, 26, 67]. Although the enzymatic activity of esterase as well as carboxypeptidase was demonstrated to be deficient in patients with galactosialidosis [68, 69], its in vivo involvement as an esterase is still unclear, and the target protein substrate for cathepsin A also remains to be clarified.

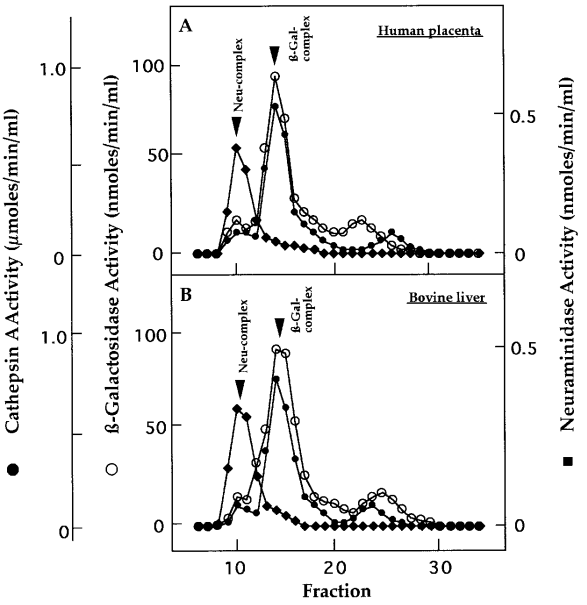

Figure 4. Coelution of neuraminidase, β-galactosidase and cathepsin A activities on HPLC. *p*-Aminophenyl thio-β-D-galactoside (PATG)-Sepharose affinity-purified β-galactosidase preparations from human placenta (*A*) and bovine liver (*B*) (each 100 μg protein) were fractionated by gel filtration of high performance liquid chromatography (HPLC) on a Shim-pack Diol-300 column (0.75×25 cm) with 20 mM acetate buffer (pH 4.5) containing 0.15 M sodium chloride. The column was eluted at 0.3 ml/min, and 160 μl fractions were collected. β-Galactosidase and neuraminidase activities were assayed using 4-methylumbelliferyl derivatives, and carboxypeptidase activity was measured using CBZ-Phe-Leu as substrates.

Cathepsin A as an essential constituent of the lysosomal β-galactosidase and neuraminiadse complexes

In the course of studies on β-galactosidase deficiency diseases, cathepsin A was found to be an essential stabilizing constituent of the lysosomal β-galactosidase and neuraminidase complexes. The protective function of cathepsin A is distinct from its multicatalytic function. This section describes the functional significance of cathepsin A in the lysosomal multienzyme complexes.

Cathepsin A and a lysosomal storage disease, galactosialidosis

β-Galactosidase [3.2.1.23] is a lysosomal hydrolase responsible for the cleavage of terminal β-galactose residues from glycoconjugates, most notably GM1 ganglioside [70]. The primary molecular defect in GM1 gangliosidosis and Morquio B syndrome is a deficiency of this enzyme. On the other hand, a hereditary disorder with β-galactosidase deficiency showing different clinical phenotypes from GM1 gangliosidosis was reported by Suzuki et al. [71] and subsequently found to be a new lysosomal storage disease on the basis of clinical and biochemical analyses by Wenger et al. [72]. The patients showed enzyme deficiency of neuraminidase as well as β-galactosidase, suggesting this genetic variant was different from other β-galactosidase deficiency diseases. The variant was then proposed to term 'galactosialidosis' [73]. The β-galactosidase activity in skin fibroblasts of galactosialidosis was partially restored by addition of leupeptin, a thiol protease inhibitor, to cells in culture [74]. Van Diggelen et al. studied turnover of β-galactosidase in fibroblasts from patients with various types of β-galactosidase deficiency diseases and demonstrated that β-galactosidase in galactosialidosis patients apparently has a reduced synthesis rate compared with normal controls [75]. However, the enzyme showed normal hydrolytic properties, suggesting an abnormal turnover rate of β-galactosidase in galactosialidosis. A human-mouse somatic cell hybrid analysis also indicated that the product of the defective gene in galactosialidosis may be involved in posttranslational processing or regulation of lysosomal β-galactosidase and neuraminidase [76].

Molecular phenotypes of β-galactosidase in β-galactosidase deficiency diseases are different from those in normal controls [77–81]. In normal fibroblasts, more than 85% of β-galactosidase was shown to exist as a stable $>$ 700-kDa multimeric form; however, it was not detected in galactosialidosis fibroblasts. Although the β-galactosidase activity in fibroblasts from galactosialidosis patients was partially restored by leupeptin, the restored β-galactosidase was in the monomeric but not multimeric form [78]. Hoogeveen et al. demonstrated the presence of a corrective factor in culture media of normal human skin fibroblasts capable of restoring the enzyme deficiencies of both β-galactosidase and neuraminidase in galactosialidosis [82]. This

corrective activity was effective only in galactosialidosis but not in either GM1 gangliosidosis or mucolipidosis I (sialidosis). As mentioned, the protective factor was subsequently found to be deficient in galactosialidosis [29] and was shown to be cathepsin A [24]. Biochemical and clinical phenotypes of galactosialidosis will be described below.

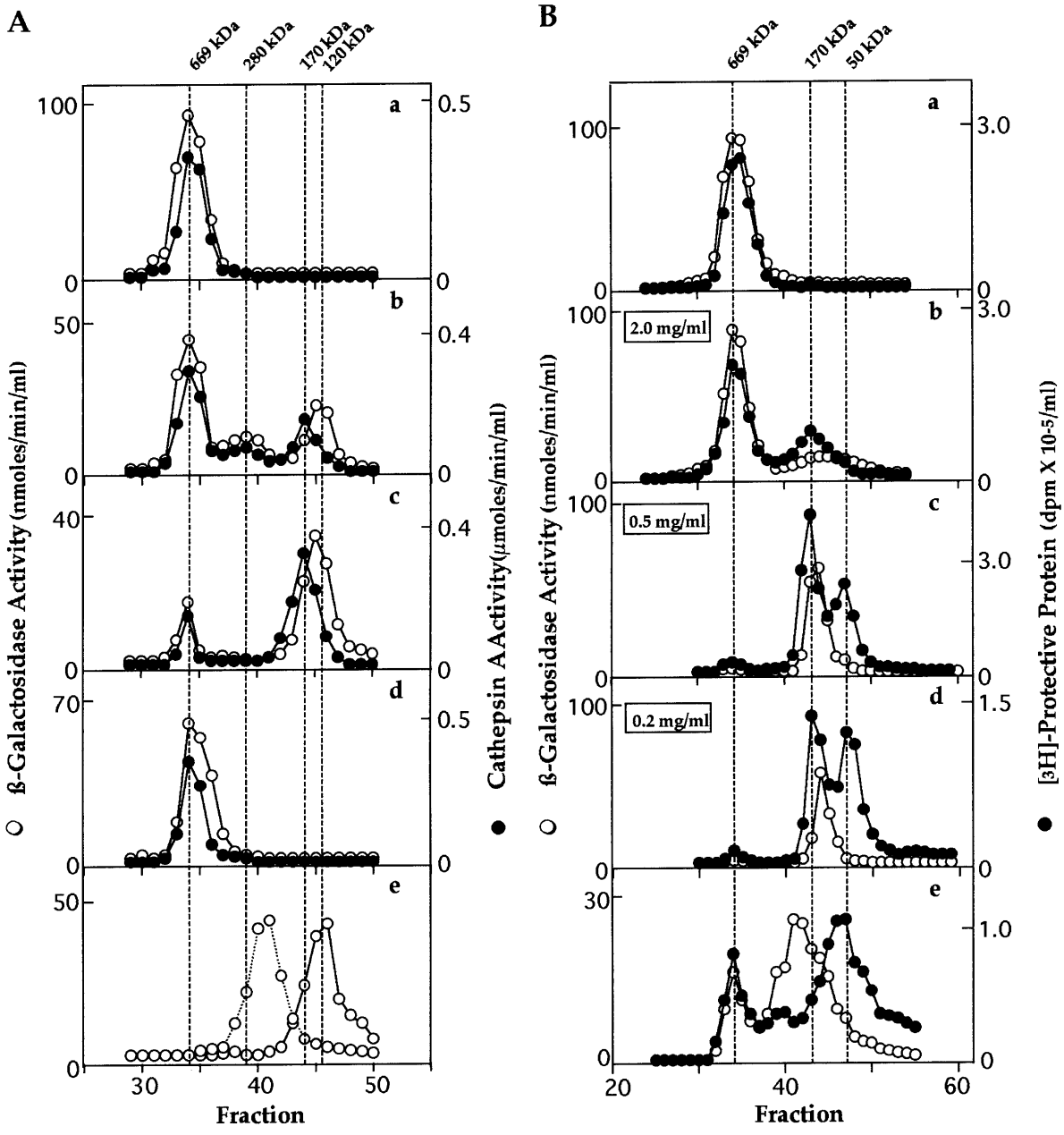

Figure 5. Molecular conversion of the bovine β-galactosidase/cathepsin A complex. (*A*) pH-dependent dissociation-association of the complex. Purified intact complex was dialyzed for up to 15 h at pH 7.5 (dissociation buffer) and a concentration of 1 mg/ml. Each sample (20 μg protein) was chromatographed by gel filtration of HPLC on a Shim-pack Diol-300 column as described [52]. [a] the intact complex; [b] after 3 h dialysis; [c] after 15 h dialysis. To assess complex reconstitution, the dissociated sample [c] or the purified 120-kDa β-galactosidase was dialyzed for 15 h at pH 4.5 (buffer A) and 1 mg/ml. [d] reconstitution of the dissociated complex [c] after 15 h redialysis; [e] β-galactosidase before (- - - - - -) and after (· · · · · · · · ·) dialysis. (*B*) Dissociation of [^{3}H]-DFP-cathepsin A from the β-galactosidase complex. The [^{3}H]-DFP complex was dialyzed for 15 h against dissociation buffer (pH 7.5) at different protein concentrations and chromatographed by gel filtration on a Shim-pack Diol-300 column [52]. [a] the intact complex before dialysis; after 15 h dialysis at 2.0 mg/ml [b], 0.5 mg/ml [c] and 0.2 mg/ml [d]; [e] reconstitution of the dissociated complex [d] after 15 h redialysis at pH 4.5 (buffer A). Buffer A: 50 mM acetate buffer (pH 4.5) containing 0.1 M NaCl. Dissociation buffer: 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl.

Cathepsin A in the β-galactosidase and neuraminidase complexes

There are accumulated reports describing molecular heterogeneity of mammalian lysosomal β-galactosidase and neuraminidase [30, 65, 80, 83–87]. In addition to mammalian enzymes, chicken lysosomal β-galactosidase and neuraminidase were also shown to occur as a multienzyme complex with cathepsin A [88]. Similar to human enzymes [84–87, 89], typical molecular features of lysosomal β-galactosidase and neuraminidase also can be seen in bovine enzymes (fig. 4). Bovine β-galactosidase and neuraminidase elute together with the enzyme activity of cathepsin A on gel filtration [52]. Immunoprecipitation experiments using antibodies against β-galactosidase and cathepsin A demonstrated that they are tightly bound multienzyme complexes. Although the neuraminidase complex depolymerizes irreversibly at pH 7.5 with inactivation of the neuraminidase activity, the β-galactosidase complex shows a pH-dependent and reversible molecular conversion [52]. At pH 7.5, the 700-kDa bovine β-galactosidase complex dissociated into a 120-kDa β-galactosidase dimer and a 170-kDa cathepsin A with formation of an intermediate 280-kDa complex (fig. 5A/b,c). After this dissociation, the 700-kDa β-galactosidase complex was reversibly reconstituted in the presence of the 170-kDa cathepsin A at pH 4.5. On the other hand, in the absence of cathepsin A, β-galactosidase was aggregated to a 260-kDa tetramer but not the multimeric form (fig. 5A/e). Interestingly, cathepsin A in the complex was shown to dissociate finally into the 50-kDa monomer that was void of the complex formation activity when the [^{3}H]-DFP-labeled complex was dissociated under diluted conditions (fig. 5B/b–d). These findings clearly demonstrate the requirement of cathepsin A dimer or oligomer (but not the monomer) for in vitro reconstitution of the β-galactosidase complex.

Stoichiometric analysis of the β-galactosidase and neuraminidase complexes provides variable insight into understanding the structure-function relationship of the complexes. The human β-galactosidase complex is composed of four molecules of β-galactosidase and eight molecules of carboxypeptidase monomers [89]. On the other hand, stoichiometry of the human neuraminidase complex is currently unknown due to a lack of information on the neuraminidase polypeptide. Recently, cDNA for human lysosomal neuraminidase has been cloned [90, 91]. An expression study of the neuraminidase cDNA in mutant human fibroblasts demonstrated that the neuraminidase activity was restored in a cathepsin A-dependent manner [90]. Further studies using a cDNA for neuraminidase will provide a more detailed picture of the molecular relationship between cathepsin A and lysosomal neuraminidase.

Table 3. Mutations of the cathepsin A gene in galactosialidosis.

Onset	Allelic mutant combination	Reference
Juvenile		
	SpDEx7/Gln21Arg	104
	SpDEx7/Ser23Tyr	105
	SpDEx7/Trp37Arg	104
	SpDEx7/Tyr367Cys	104, 106
	SpDEx7/SpDEx7	106, 107
Late-infantile		
	Tyr221Asn/+1 frame shift	105
	Tyr221Asn/?	105
	Phe412Val/Tyr221Asn	105
	Phe412Val/Met378Thr	105
	Phe412Val/Phe412Val	105
	Different two frameshifts	108
Early-infantile		
	Val104Met/Leu208Pro	105
	Tyr367Cys/Ser62Leu	104
	Tyr367Cys/Tyr367Cys	109
	Tyr367/Cys/?	104
	Gly411Ser/?	105

Saposin B, a sphingolipid activator protein that stimulates the enzymic hydrolysis of GM1 ganglioside by lysosomal β-galactosidase [92], was shown to stabilize recombinant human cathepsin A in vitro [25]; however, whether saposin B stabilizes cathepsin A in vivo is still unknown.

Galactosialidosis and activation of human lysosomal neuraminidase

Human lysosomal neuraminidase in a partially purified preparation is activated by brief incubation under acidic pH condition [84, 93–96]. The activation occurs in a time- and pH-dependent fashion, suggesting involvement of certain enzymatic modification(s) in the activation process. Activation of neuraminidase did not occur in a liver sample of a patient with galactosialidosis [96], raising a possibility that the deficiency of the cathepsin A activity might have influenced the activation process. A recent study demonstrated that neuraminidase bound to cathepsin A precursor, shortly after its synthesis, was transported to lysosomes. Interestingly, translocation was not demonstrated with mutant cathepsin As [97]. These findings suggest that cathepsin A may have a novel function as a lysosomal transporter protein, implicating a connection between the translocation process and the activation process of lysosomal neuraminidase.

Gene mutations in galactosialidosis

Galactosialidosis is an autosomal recessive lysosomal storage disease caused by a molecular defect of lysoso-

mal cathepsin A [98–101] with a high incidence in the Japanese population [102]. There are three types of onset that induce different clinical manifestations: early-infantile, late-infantile and juvenile/adult onset. The early-infantile form shows the most severe clinical manifestations, including massive edema, skeletal dysplasia and cherry-red spots [99, 103]. There are more than 10 different mutations identified in the cathepsin A gene of patients with galactosialidosis (table 3). Patients are generally compound heterozygotes of two different mutations. A number of mutant allelic combinations give rise to heterogeneity in clinical manifestations; however, the following evidence illustrates a correlation between the biochemical and clinical phenotypes and the genotype of galactosialidosis.

Mutations in the juvenile form (SpDEx7 mutation)

A unique and common mutation in the cathepsin A gene was recently found in Japanese patients with galactosialidosis, a common splice junction mutation at the 5′ splice donor site of the intron 7 (SpDEx7) causing skipping of exon 7 in the cathepsin A gene termed 'SpDEx7' [110]. This genotype has been found in the juvenile onset form, and a correlation between the clinical phenotype and SpDEx7 genotype has been suggested [107]. Patients who carried no SpDEx7 were all early-infantile onset with the most severe clinical manifestations, whereas homozygotes of SpDEx7 showed less severe manifestations than heterozygotes. Patients with the SpDEx7 genotype show milder clinical phenotypes than those with other allelic mutant combinations.

Mutations in the late-infantile form

The largest number of patients have late-infantile onset within 6 to 12 months after birth [99]. Briefly, the patients show progressive neural degeneration with mild deterioration and mental retardation and survive into adulthood. Recent case reports of a Japanese patient described clinical and biochemical phenotypes of the late-infantile form in detail [81, 111–113]. Mutations at the ^{412}Phe and ^{221}Tyr residues are typical abnormalities for the late-infantile form of the disease. Whether these mutations are present together or with other mutations appears to underlie the clinical phenotype in the late-infantile form. Both ^{221}Tyr and ^{412}Phe mutants produced the mature 32-kDa subunit of cathepsin A [104, 105], but the ^{221}Tyr mutant protein expressed a higher carboxypeptidase activity than the ^{412}Phe mutant. Consistent with differences in clinical severity, the ^{412}Phe mutant shows more clinically severe manifestations than the ^{221}Tyr mutant. The ^{412}Phe mutation was demonstrated to impair dimerization of cathepsin A [114]. Dimerization with the counterpart subunit creates interfaces required for complex formation with β-galactosidase [65]. The interface consists of two sequences in the core domain, ^{76}Gln–^{84}Tyr and ^{400}Val–^{407}Glu (fig. 1), and the mutation at ^{412}Phe seems to alter steric conformation of the interface sequences, resulting in impaired dimerization.

Mutations in the early-infantile form

The clinical course of the early-infantile form of galactosialidosis is progressive, with rapid deterioration resulting in death within several months after birth [99]. There have been only a few descriptions of the early-infantile form; however, recent case reports of an Italian patient with a mutant allelic combination of Val104Met/Leu208Pro described clinical and pathological abnormalities of the infantile form in detail [103, 105]. A different mutation causing the early-infantile phenotype has also been identified in a Japanese family [109]. The patients were homozygotes for the Tyr367Cys substitution. The mutant gene expressed neither the cathepsin A precursor nor the mature form in transfected cells, reflecting clinical severity of the patients.

As mentioned, a variety of the cathepsin A gene mutations cause multiple phenotypes of galactosialidosis. A three-dimensional structure analysis of 11 different mutant cathepsin As suggested that those mutations induced drastic changes in the folding and stability of cathepsin A and resulted in simultaneous disruption of its function [115]. In addition to structural analysis, it will be necessary to investigate the molecular interaction of mutant cathepsin A with β-galactosidase and neuraminidase in order to more fully understand the correlation between the biochemical and clinical phenotypes and the genotype of galactosialidosis.

Therapeutic potential for human galactosialidosis

Cathepsin A knockout mice were recently generated [31]. These mutant mice showed clinical symptoms similar to human patients with galactosialidosis. Transplanting erythroid precursor cells overexpressing human cathepsin A into the mutants corrected the deficient phenotype [31]. Moreover, very recently, bone marrow-derived macrophages overexpressing human cathepsin A were also effective in correcting the deficient phenotype of the mutant mice [116]. The expression of cathepsin A by the transgenic macrophages occurred in all organs including the central nervous system. Although some neural cells showed no improvement of the deficient phenotypes after transplantation, these findings

opened the possibility to develop future therapeutic strategies for human patients with galactosialidosis.

Future aspects

During the last decade, research on cathepsin A has made great progress with the discovery of a genetic disorder due to its mutation. The most significant findings point to its role in stabilization of lysosomal β-galactosidase and neuraminidase and its deficiency in the fatal human disease called galactosialidosis. Multiple mutations of the cathepsin A gene that cause different clinical phenotypes were identified in patients with galactosialidosis. Although cathepsin A protects β-galactosidase and neuraminidase from intralysosomal proteolysis by forming a high molecular weight complex, it is not clear how this enzyme complex is assembled. In order to understand the molecular construction of the complex, identification of interface sequences present on each enzyme will be necessary. Furthermore, achieving in vitro reconstitution of the complex under controlled conditions will provide further insight into the structure-activity relationship of the complex. Lacking either β-galactosidase or neuraminidase alone seems not to interfere with complex formation. For example, β-galactosidase in sialidosis and neuraminidase in GM1 gangliosidosis are functionally active, raising a simple question of how, in these disorders, a functionally active complex forms without one of these enzymes. Cathepsin A is possibly involved in terminal processing of β-galactosidase and neuraminidase within the complex; however, the targeted protein substrates in vivo remain unknown.

Cathepsin A knockout mice have been recently described and have contributed further insights into understanding biochemical and clinical features of galactosialidosis. Studies on correction of the deficient phenotypes in mutant mice will provide strategies for therapeutic applications to human galactosialidosis. Current evidence also implicates cathepsin A in regulation of a variety of small bioactive peptides. However, no pathological or clinical consequences associated with dysfunction of bioactive peptide function have been studied in the cathepsin A knockout mice. Further investigation of the mutant mice will clarify the function of cathepsin A in regulation of bioactive small peptides in vivo.

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