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# The crystal structure of a Cys25→Ala mutant of human procathepsin S elucidates enzyme–prosequence interactions

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## Abstract

The crystal structure of the active-site mutant Cys25→Ala of glycosylated human procathepsin S is reported. It was determined by molecular replacement and refined to 2.1 Å resolution, with an *R*-factor of 0.198. The overall structure is very similar to other cathepsin L-like zymogens of the C1A clan. The peptidase unit comprises two globular domains, and a small third domain is formed by the N-terminal part of the prosequence. It is anchored to the prosegment binding loop of the enzyme. Prosegment residues beyond the prodomain dock to the substrate binding cleft in a nonproductive orientation. Structural comparison with published data for mature cathepsin S revealed that procathepsin S residues Phe146, Phe70, and Phe211 adopt different orientations. Being part of the S1' and S2 pockets, they may contribute to the selectivity of ligand binding. Regarding the prosequence, length, orientation and anchoring of helix  $\alpha$ 3p differ from related zymogens, thereby possibly contributing to the specificity of propeptide–enzyme interaction in the papain family. The discussion focuses on the functional importance of the most conserved residues in the prosequence for structural integrity, inhibition and folding assistance, considering scanning mutagenesis data published for procathepsin S and for its isolated propeptide.

**Keywords:** crystal structure; cysteine proteinase; procathepsin S; propeptide

Cathepsin S (EC 3.4.22.27) is a cysteine protease of the papain family (clan C1A) (Kirschke et al. 1995). In mammals, papain-like proteases are mainly involved in

lysosomal protein degradation. Cathepsin S, first mentioned by Turnsek et al. (1975), has been described in detail by Kirschke et al. (1986). Like the related cathepsins L and K, it is an endopeptidase, but, in contrast to them, it is stable at neutral pH. Cathepsin S has been found almost exclusively in antigen-presenting cells, B lymphocytes, and dendritic cells, where it is involved in processing of the invariant chain of the MHC II complex (Riese et al. 1996). Selective inhibition of cathepsin S results in a delayed immune response (Riese et al. 1998). The enzyme is probably also necessary for normal retinal function, as suggested by adenovirus-mediated antisense gene transfer experiments in rats (Lai et al. 2000).

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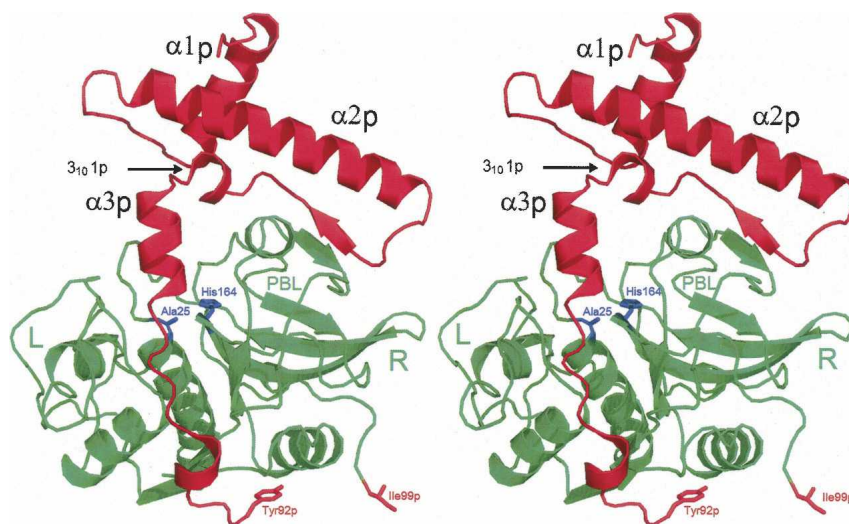
*Abbreviations:* RMSD, root-mean-square deviation; PBL, prosegment binding loop; Residues and atoms have been named as in the Protein Data Bank entry (e.g., Met68p<sup>CE</sup>, C $\epsilon$  of residue methionine 68p).

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The structure of mature cathepsin S is known from X-ray data obtained with a Cys25 → Ser mutant (Turkenburg et al. 2002). It is a monomer with two domains (Fig. 1, L and R, left and right in the standard view). The interface of the two domains provides the structure for the active-site cleft, comprising the substrate-binding subsites (Schechter and Berger 1967) and the catalytic triad. The catalytic His164 and the stabilizing Asn184 are part of the polar surface of the R domain, whereas the L domain contributes the catalytic Cys25. The cathepsin S structure is not only very similar to that of the related mammalian enzymes, e.g., cathepsins K, L, and H, but also to the prototype of clan C1A, the plant enzyme papain (Turkenburg et al. 2002). As most other peptidases, cathepsin S derives from an inactive precursor by auto- or heterocatalytic processing. In the zymogen, the N terminus of the catalytic unit bears a 99 amino acid extension. Procathepsin S has been classified as a cathepsin L-like endopeptidase, one of three subfamilies in the papain clan (Karrer et al. 1993; Kirschke et al. 1995; Wex et al. 2000). The structures of two zymogens of this subfamily, procathepsins L (Coulombe et al. 1996) and K (LaLonde et al. 1999; Sivaraman et al. 1999), have been determined by X-ray analysis. It was found that there is no difference in the structure of the catalytic units between enzymes and zymogens, i.e., the catalytic power is already present in the zymogen, but nearly completely masked by the prosegment. Part of this prosequence occupies the active site cleft in the reverse nonproductive orientation. This inhibitory mechanism is not restricted to

the cathepsin L-like subfamily, but was also found for cathepsins B and X, which feature much shorter prosequences (Sivaraman et al. 2000; Quraishi and Storer 2001). The prosegment structures of the two cathepsin L-like subfamily members known so far are similar. The N-terminal part forms a small domain (P-domain), whose backbone is built by two crossed helices,  $\alpha 1p$  and  $\alpha 2p$ . The P-domain anchors in the corresponding binding loop of the R-domain and forces the adjacent extended part of the prosequence into the active site cleft (Coulombe et al. 1996; LaLonde et al. 1999; Sivaraman et al. 1999).

The inhibitory function of the prosegment has been intensely studied *in vitro* with isolated propeptides, revealing that they are competitive tight binding inhibitors of their mature enzymes (Fox et al. 1992; Taylor et al. 1995; Carmona et al. 1996; Volkel et al. 1996; Maubach et al. 1997). Inhibition studies with cognate, noncognate, and even chimeric propeptides of the closely related cathepsins S, L, and K, revealed that there is some selectivity, but not an absolute specificity (Carmona et al. 1996; Maubach et al. 1997; Guo et al. 2000). The crystallization of a cathepsin–propeptide complex has not yet been reported. Therefore, the orientation of an isolated inhibitory propeptide in the active site can only be suggested. As achieving selectivity is a challenge in the design of cysteine protease inhibitors with pharmaceutical significance, structural data on prosequence–enzyme interactions are of significant value (Chowdhury et al. 2002).



**Figure 1.** Overall structure of the human procathepsin S Cys25 → Ala mutant. The amino acid sequence of the molecule comprises 316 residues (including the N-terminal methionine), of which 308 were visible in the electron density, 91 for the prosegment (in red, suffix “p”) and 217 for the catalytic unit (in green). The gap at the C-terminal end of the prosegment (Tyr92p–Ile99p) results from poorly defined electron density in this region. For clarity, only some of the secondary structure elements ( $\alpha 1p$ ,  $\alpha 2p$ ,  $3_{10}1p$ , and  $\alpha 3p$ ) of the prosequence are labeled. “L” and “R” specify the left and right domain of the catalytic unit. “PBL” is the prosegment-binding loop. The C terminus (Ile217) and the active site residues (Ala25 and His164) are indicated. Stereo picture prepared with PyMOL (DeLano 2002).

Further interest came from the discovery of prosegment-dependent folding of cathepsin L-like subfamily members, as discussed for the first time by Smith and Gottesman (1989), but proved only a decade later (Yamamoto et al. 1999; Wiederanders 2000). Identification of a missense mutation in the prosequence of cathepsin K in a patient with pycnodysostosis, resulting in a misfolded, inactive protein (Hou et al. 1999), illustrates the clinical relevance. One can assume that the prosequence forms a well-structured template, stabilizing a critical transition state of the catalytic unit in an enzyme-like manner. This lowers the energy barrier along the folding-reaction coordinate. Considering such prosequences as single-turnover catalysts, they have been termed foldases (Zhu et al. 1989).

The consequences of point mutations of highly conserved cathepsin S prosequence residues on refolding and inhibition have been studied systematically (Schilling et al. 2001). These results will be discussed in the light of the 2.1 Å X-ray structure of the Cys25→Ala procathepsin S mutant presented here.

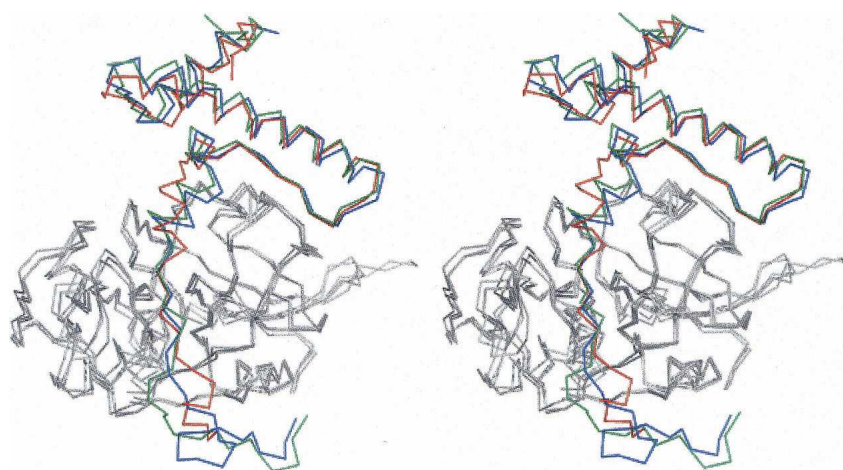
## Results

### *Quality of the structural model and overall structure*

The asymmetric unit of the crystals contains one molecule of the Cys25→Ala procathepsin S mutant and 242 water molecules. The RMSD of bond lengths and angles of the refined structural model are 0.009 Å and 1.35°, respectively, indicative of good steric geometry. A Ramachandran plot (Ramachandran and Sasisekharan 1968) had 86.1% of the residues in the most favored region and 13.5% of the residues in the additionally

allowed regions. No residues were found in the disallowed region, and only one residue (Tyr92p) adopted conformational angles in the generously allowed region. The published amino acid sequence (99 residues in the prosegment, marked with suffix p, and 217 in the catalytic domain) (Shi et al. 1992; Wiederanders et al. 1992) fits well into the electron density, with the exception of a few solvent-exposed residues at both ends of the prosequence, Ala1p–Gln2p and Lys93p–Arg98p at the N and C terminus, respectively. As electron density was poorly defined, these residues are very likely disordered in the crystal and, consequently, were omitted from the structural model (Fig. 1). Procathepsin S was crystallized in the Asn89p-glycosylated form, as indicated by the shape of the electron density extending from this side chain, but the difference electron density map did not provide insight into details of the glycosidic moiety. Structural integrity of the mutant zymogen was proven by SDS-PAGE; additional bands due to autocatalytic cleavage have not been observed.

As can be seen in Figure 2, the overall fold of procathepsin S is very similar to that of the human cathepsin L-like subfamily members elucidated so far, procathepsins K and L (PDB 1BY8 and PDB 1CS8, respectively) (Coulombe et al. 1996; LaLonde et al. 1999; data not shown) to procaricain from *Carica papaya* (PDB 1PCI) (Groves et al. 1996). Regarding the overall structure of the catalytic unit (McGrath et al. 1998; Turkenburg et al. 2002; Ward et al. 2002; Pauly et al. 2003), there is no difference between the zymogen and the mature enzyme, just as observed previously for cathepsins L and K when compared to their proenzymes (Coulombe et al. 1996; LaLonde et al. 1999; Sivaraman et al. 1999). Superposition of mature cathepsin S (PDB 1GLO), (Turkenburg et al.



**Figure 2.** Superposition of the C $\alpha$  tracing of procathepsin S (red), K (blue), and L (green), as taken from PDB entries 2C0Y, 1BY8, and 1CS8, respectively. Only the prosequences are drawn in colors, the catalytic units are gray. The superposition underlines the high similarity for procathepsin S with procathepsin K (RMSD 0.79 Å) and procathepsin L (RMSD 0.88 Å). Stereo picture prepared with PyMOL (DeLano 2002).

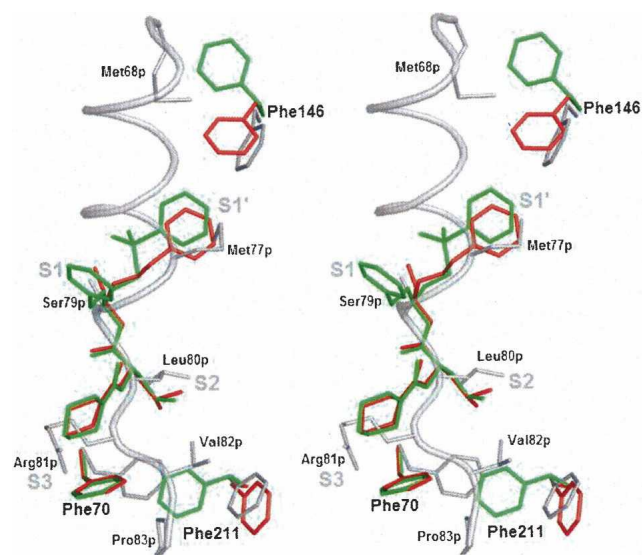
2002) onto the catalytic unit of procathepsin S (this study) results in an RMSD on C $\alpha$ s of only 0.36 Å with no outliers (largest deviation 1.36 Å).

Due to occupation by part of the prosegment, subsite structures in the proenzyme are different in some details from those in mature cathepsin S in complex with various inhibitors (PDB entries 1MS6 and 1NPZ) (Ward et al. 2002; Pauly et al. 2003). As shown in Figure 3, the superposition of the structures reveals different orientations of the side chains of phenylalanines 70, 146, and 211.

### Structure of the prosegment

The N-terminal part of the prosegment forms the small P-domain, comprising three  $\alpha$ -helices ( $\alpha$ 1p=10p–20p,  $\alpha$ 2p=29p–52p,  $\alpha$ 3p=70p–76p) and one  $\beta$ -strand ( $\beta$ 1p=57p–60p) (Fig. 1).  $\alpha$ 2p and  $\beta$ 1p are part of a hairpin structure. The side chains of Leu9p, Trp13p, Trp16p, Ile36p, Trp37p, Leu41p, Met62p, and the aliphatic part of Glu38p contribute to the hydrophobic core of the P-domain. Helix  $\alpha$ 1p, bearing Trp13p and Trp16p, and helix  $\alpha$ 2p with Trp37p are connected by a seven-residue loop. They intersect at an angle of  $\sim 65^\circ$ , so that in the center of the core, the aromatic rings of the specified tryptophans are positioned perpendicular to each other (Fig. 4). Outside the core, hydrogen bonds and salt bridges, listed in Table 1, contribute to the stabilization of

the P-domain. The salt bridges Lys22p–Asp67p and Arg33p–Glu72p are fixing the  $\alpha$ 3p-helix to the scaffold of the P-domain (Fig. 4). Amino acids contributing to the P-domain's stability are well conserved; sequence identity of the propeptides in the cathepsin L-like subfamily is between 26% and 58% (Karrer et al. 1993; Coulombe et al. 1996; Groves et al. 1998). Not only the primary, but also the tertiary structure of the prosegments of the cathepsin L-like subfamily members is very similar, as illustrated in Figure 2 by main chain superposition of procathepsins S, K, and L. In all these zymogens, the prosegments embrace the catalytic domains like a clamp. However, Figure 2 also reveals differences. The  $\alpha$ 3p-helix in procathepsin S is rotated within the paper plane by  $\sim 20^\circ$  counterclockwise compared to procathepsins K and L (and  $\sim 10^\circ$  clockwise to procathepsin B; data not shown). The consequence is an additional  $3_{10}$ -helix ( $3_{10}$ 1p=64p–67p) between  $\beta$ 1p and  $\alpha$ 3p, and larger distances between some  $\alpha$ 3p residues (Val73p, Ser75p, Leu76p, and Met77p) and the primed subsites in procathepsin S than in the related zymogens (Figs. 1,2,5). Beyond the C terminus of helix  $\alpha$ 3p, the prosegment adopts an extended conformation and follows the substrate-binding cleft, in a direction reverse to substrate orientation. A water molecule fills the cavity created by the catalytic site mutation (Cys 25 $\rightarrow$ Ala), forming hydrogen bonds with the side chains of Gln19 and His164 of the catalytic unit and with Ser79p, and  $\alpha$ 3p residue (Fig. 6).

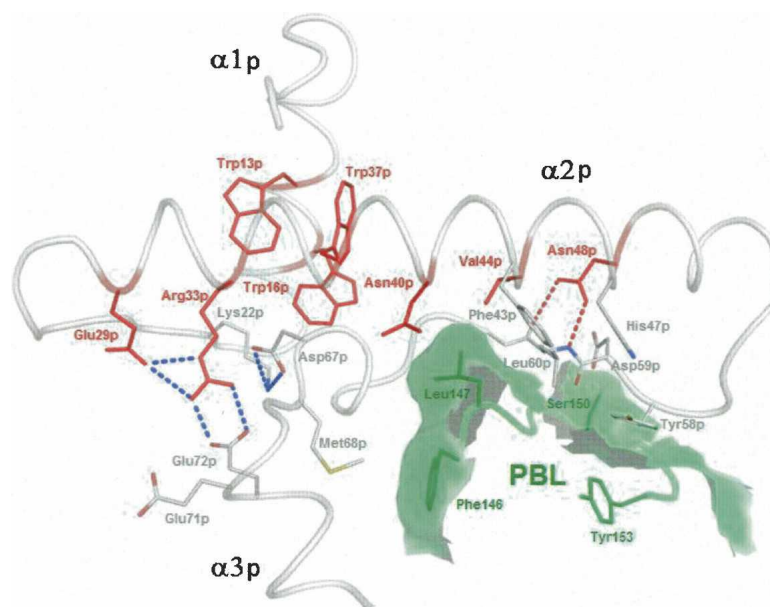


**Figure 3.** Flexibility of Phe146, Phe70, and Phe211 lining the substrate binding cleft, illustrated by superposition of procathepsin S and two cathepsin S/inhibitor complexes. (Gray) procathepsin S (this work); (red) cathepsin S/morpholine-4-carboxylic acid [1S-(2-benzyloxy-1R-cyanoethylcarbamoyl)-3-methyl butyl] amide complex (PDB entry 1MS6); (green) cathepsin S/4-morpholinecarbonyl-Phe-(S-benzyl)Cys- $\psi$  (CH=O) complex (PDB entry 1NPZ). Stereo picture of superposition prepared with PyMOL (DeLano 2002).

### Interactions between the prosegment and the catalytic unit

At first glance, the orientation of the cathepsin S prosegment relative to the catalytic unit seems to be very similar to what is seen in the procathepsins L, K, and B. In all these papain family members, the catalytic unit contacts the prosegment via two regions, i.e., the prosegment binding loop (PBL) and the substrate binding cleft (S' and S subsites). Nevertheless, detailed analysis reveals some peculiarities of these two regions in procathepsin S. The following interactions are observed:

1. Residues of the  $\alpha$ 2p helix and the  $\beta$ 1p strand pack against the platform of the PBL, a large omega loop comprising residues Pro143–Glu155. On one side of this loop, three residues of the  $\beta$ 1p strand are involved in a conserved hydrophobic–hydrophilic–hydrophobic interaction profile: the side chains of Tyr58p, Asp59p, and Leu60p contact the side chains of Tyr153, Ser150, and Phe43p/His47p/Tyr58p, respectively (Fig. 4). This is a structural feature conserved in the subfamily, as in procathepsin K Tyr56p, Glu57p and Leu58p contact Tyr150, Lys147, and Tyr41p/His45p/Tyr56p and in procathepsin L Phe56p, Thr57p, and Met58p contact Tyr151, Glu148 (main chain), and Met41p/His45p/Phe56p. On



**Figure 4.** Conserved residues involved in stabilization of the procathepsin S P-domain. Members of the ER(F/W)N(I/V)N motif are labeled in red. Dashed red lines symbolize hydrogen bonds, and blue lines symbolize salt bridges. Residues of the prosegment binding loop (PBL) of the enzyme are printed in dark green, the calculated contact area of PBL with the prosequence is shadowed in light green. Picture prepared with PyMOL (DeLano 2002).

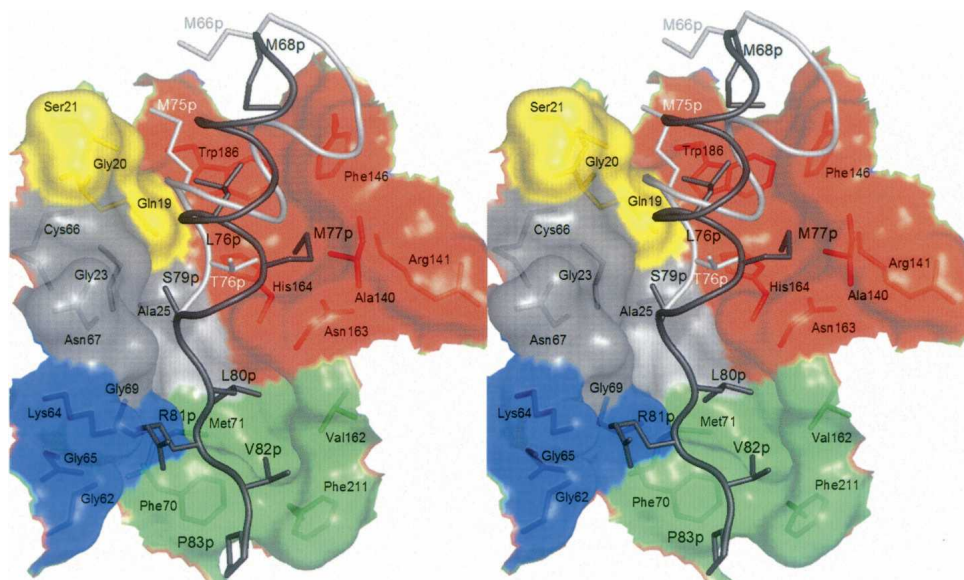
the other side of the PBL, Phe146 makes hydrophobic contacts to a cluster of residues mainly from helix  $\alpha 3p$ : Leu65p, Met68p, Val73p, Leu76p, and Met77p. Helix  $\alpha 3p$  is differently oriented in procathepsin S compared to other procathepsins. This cannot be explained by altered hydrophobicity, as the hydrophobic character of this region is conserved for all residues except the last (procathepsin K: Leu63p, Met66p, Val71p, Met75p, and Thr76p; procathepsin L: Phe63p, Met66p, Phe71p, Met75p, and Asn76p).

2. The S1' pocket, occupied by the last residue of  $\alpha 3p$ , Met77p, is formed by the hydrophobic moieties of Ala140, Arg141, Phe146, His164, and Trp186 (Fig. 5). Phe146 does not only border the S1' pocket, but also contributes to an adjacent poorly defined pocket, distal from S1'. McGrath et al. (1998) assume that Phe146 could bind a P2' residue, but the inhibitor in their structure does not provide an equivalent moiety. This may also be the reason for Turkenberg et al. (2002) not to predict more than one primed subsite in mature cathepsin S.

**Table 1.** Stabilizing salt bridges and hydrogen bonds within the proregion

Atom	Distance (Å)	Atom	Distance (Å)	Atom	Distance (Å)	Atom
		Asp6p <sup>N</sup>	2.8	Glu38p <sup>OE1</sup>		
		Asp6p <sup>O</sup>	2.9	Arg34p <sup>NH2</sup>		
		Asp10p <sup>OD1</sup>	2.9	Arg49p <sup>NH1</sup>		
		Trp13p <sup>NE1</sup>	2.8	Glu30p <sup>OE2</sup>		
		Lys17p <sup>NZ</sup>	3.0	Glu30p <sup>OE2</sup>		
		Lys22p <sup>NZ</sup>	2.9	His64p <sup>O</sup>		
Tyr24p <sup>OH</sup>	2.6	Asp67p <sup>OD1</sup>	3.5	Lys22p <sup>NZ</sup>	3.3	Asp67p <sup>OD2</sup>
		Arg33p <sup>NH2</sup>	3.3	<b>Glu29p<sup>OE1</sup></b>	3.1	<b>Arg33p<sup>NE</sup></b>
		Arg33p <sup>NH1</sup>	2.8	Glu72p <sup>OE1</sup>		
		Arg33p <sup>NH2</sup>	2.8	Glu72p <sup>OE2</sup>		
Arg34p <sup>NE</sup>	2.9	Glu38p <sup>OE1</sup>	3.2	Arg34p <sup>NH2</sup>		
		Asn40p <sup>ND2</sup>	2.8	Gly66p <sup>O</sup>		
		Asn48p <sup>OD1</sup>	2.9	Leu60p <sup>N</sup>		
		Asn48p <sup>ND2</sup>	2.9	Leu60p <sup>O</sup>		
		Glu50p <sup>OE1</sup>	2.8	His56p <sup>NE2</sup>		

Conserved residues belonging to the ER(F/W)N(I/V)N-motif are highlighted in gray. Salt bridges are shadowed in gray.



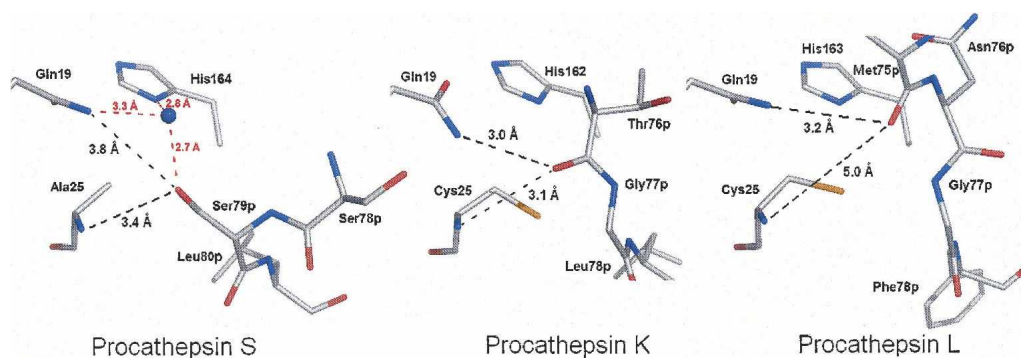
**Figure 5.** Interaction of the prosegment (cathepsin S, dark gray; cathepsin K, light gray; color code for numbering and chain) with the active site cleft of cathepsin S. The subsites are colored as follows: S2', yellow; S1', red; S1, gray; S2, blue; and S3, green. Stereo picture prepared with PyMOL (DeLano 2002).

However, the zymogen structure permits differentiation between S1', faced by Met77p, and S2', faced by Leu76p (Fig. 5). Crystal structures of procathepsins L and K revealed S2' pockets, occupied by Met75p, as extensions of the crevice formed by the tryptophans 189 and 184, respectively, occupied by Met75p (Coulombe et al. 1996; LaLonde et al. 1999; Sivaraman et al. 1999). In procathepsin S, the shorter side chain of Leu76p, which substitutes for Met75p, is in van der Waals contact with Trp186, but does not completely fill S2' (Fig. 5). Thus, not all sides of the S2' pocket are well defined.

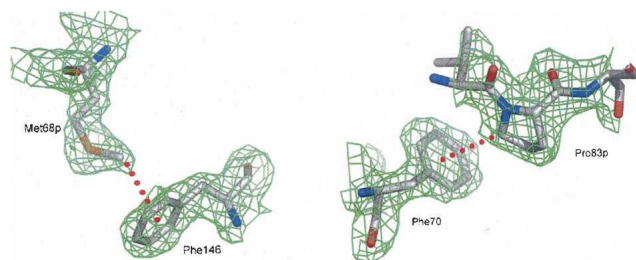
3. Another relevant contact on the S' side is the C—H... $\pi$  interaction of Met68p<sup>CE</sup> as the H-donor with the

aromatic ring of Phe146 as the acceptor (Figs. 5, 7A) (Brandl et al. 2001). This interaction is unique within the cathepsin L-like subfamily, as the position of Phe146 is occupied by Leu144 in cathepsin L and by Gln143 in cathepsin K. Therefore, methionines 66p in the procathepsins L and K, corresponding to Met68p in procathepsin S, rotate away and are solvent exposed.

4. The last residue of helix  $\alpha$ 3p and the extended part of the prosegment (Ser78p–Pro83p) interact with the non-primed subsites. As detailed in Figure 5, side chain and main chain atoms of Ser78p and Ser79p are in van der Waals contact with the S1 pocket. Compared to procathepsins K and L, helix  $\alpha$ 3p of procathepsin S is



**Figure 6.** Prosegment interaction with the active site. (Black dashed lines) Coordination with main chain nitrogen of residue 25 and side chain nitrogen of Gln19 of the oxyanion hole. Procathepsin S, (red dashed lines) coordination of an additional water molecule (blue ball) filling the void created by the active site mutation Cys25 → Ala. The side chain of Ser79p projects into the oxyanion hole. Procathepsin K, the main chain oxygen of Thr76p reaches the oxyanion hole. Procathepsin L, the main chain oxygen of Met75p is closest to the oxyanion hole. Picture prepared with PyMOL (DeLano 2002).



**Figure 7.** C–H... $\pi$  interactions between prosequence and active-site cleft residues of cathepsin S. Prosequence residues are marked with “p.” The  $2F_o - F_c$  electron density is contoured in green at a level of  $1\sigma$ . Red dashed lines symbolize C–H... $\pi$  interactions: Met68p<sup>CE</sup>...Phe146p <sup>$\pi$</sup>  (distance = 4.02 Å; *left*) and Pro83p<sup>CD</sup>...Phe70p <sup>$\pi$</sup>  (distance = 3.54 Å; *right*). Pictures prepared with PyMOL (DeLano 2002).

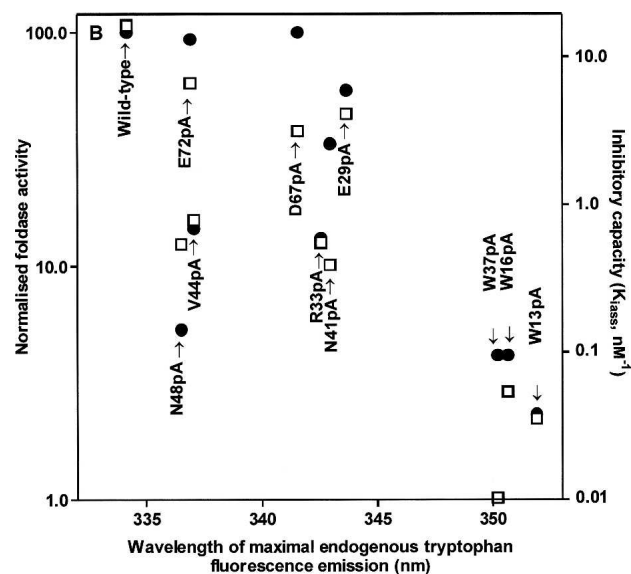
extended by Ser78p. The side chain hydroxyl group of Ser79p fills the oxyanion hole, formed by Ala25<sup>N</sup> and Gln19<sup>NE2</sup>. This mode of interaction is completely different from the situation in the procathepsins K and L, where the highly conserved oxyanion hole is occupied by the carbonyl oxygens of Thr76p and Met75p, respectively. This unique structural feature of procathepsin S is of local nature, as the adjacent Leu80p is well aligned with Leu78p and Phe78p in the procathepsins K and L, respectively (Fig. 6).

- Analogous to the other procathepsins, Leu80p projects into the S2 pocket and Arg81p into the S3 pocket. Beyond this, Pro83p<sup>CD</sup>, formally in position P5, forms a C–H... $\pi$  bond (Brandl et al. 2001) with the solvent-exposed Phe70 (Fig. 7B) at the distal end of the P2 pocket. The position of Phe70 required for this interaction is only found in the proenzyme and forces Phe211 to swing out toward the bulk solvent.

## Discussion

The overall structure of procathepsin S is very similar to that of the two other cathepsin L-like subfamily members investigated so far, procathepsins L and K (Fig. 1) (Coulombe et al. 1996; LaLonde et al. 1999; Sivaraman et al. 1999). The active-site cleft between the two domains of the catalytic unit is already preformed in the proregion, which covers the catalytic center in a non-productive orientation and anchors via residues Ser78p–Pro 83p in the nonprimed subsites. The larger part of the prosequence forms a small domain with the crossed helices  $\alpha 1p$  and  $\alpha 2p$  as scaffold. The P-domain contacts the prosegment-binding loop and the primed subsites of the catalytic unit via residues Tyr58p–Leu60p and the hydrophobic cluster Leu65p, Met68p, Val73p, Leu76p, and Met77p, respectively.

About one decade ago, motifs of conserved amino acids, e.g., ER(F/W)N(I/V)N (Karrer et al. 1993) and GNFD (Vernet et al. 1995), were identified in the prosequences of papain family members; in fact, the first of these is the basis for the recent subfamily classification (Wex et al. 2000). Conservation suggests functional and/or structural importance. The role of 10 conserved prosegment residues in procathepsin S has been probed by alanine scanning, both in vitro (Schilling et al. 2001; Pietschmann et al. 2002) and in vivo (Kreusch et al. 2000). Pietschmann et al. (2002) correlated the structural integrity of recombinant propeptide mutants, scaled by the red shift of the endogenous tryptophan fluorescence, with two basic functions, inhibition and folding, of the parental enzyme. These results are re-presented in Figure 8 because they are essential for the discussion of the functional importance of some structural features described in this paper. It can be seen that there is a rough reciprocal correlation between structural break down of the mutant P-domain and the functional parameters. Pulse-chase experiments in HEK-cells with zymogens, bearing the same mutations, confirmed this trend (Kreusch et al. 2000). This favors the hypothesis, based on physicochemical studies, that isolated propeptides, at least in complex with the corresponding enzymes, have the same fold as the proregions in the maternal zymogens (Maubach et al. 1997;



**Figure 8.** Effect of scanning mutagenesis of highly conserved prosequence residues on human cathepsin S propeptide function; “XnpA” means that amino acid X in position *n* of the propeptide is substituted by Ala. Propeptide mutants have been expressed, purified, and characterized as published by Kreusch et al. (2000). Renaturation rates (*left* ordinate, ●) are from Pietschmann et al. (2002). Inhibitor constants (*right* ordinate, □) and red shift of the endogenous tryptophan fluorescence, standing for compactness of tertiary structure (*abscissa*), are from Schilling et al. (2001).

Jerala et al. 1998; Ogino et al. 1999). Autonomous folding of the isolated cathepsin S propeptide was also shown by crystallization (Kaulmann et al. 2003). Consequently, the *in vitro* results with isolated cathepsin S propeptide mutants can be interpreted in the light of the zymogen structure.

#### *Tryptophans 13p, 16p, and 37p stabilize the core of the prodomain scaffold*

The tryptophans 13p, 16p, and 37p form a cluster at the intersection point of the helices  $\alpha$ 1p and  $\alpha$ 2p (Fig. 4), thereby stabilizing the core of the prodomain through hydrophobic interactions. The integrity of this cluster is of outstanding importance for the P-domain structure. This is deduced from two experimental series, where each of these tryptophan residues was alanine-substituted by site-directed mutagenesis. Figure 8 illustrates that tryptophan fluorescence emission spectra of only three propeptide mutants, Trp13p $\rightarrow$ Ala, Trp16p $\rightarrow$ Ala, and Trp37p $\rightarrow$ Ala, show a strong red shift (Schilling et al. 2001). The three analogous zymogen mutants, when expressed in HEK cells, were degraded by the quality control machinery of the cell (Kreusch et al. 2000). All the other mutants specified in Figure 8, including those involved in salt bridges and hydrogen bonds (e.g., Glu29p, Arg33p, Asn48p, Asp67p, and Glu72p; cf. Table 1), behaved more similarly to the wild-type control, *in vitro* as well as *in vivo*. Thus, the contribution of hydrophobic interactions between the three aromatic core residues to P-domain stability significantly exceeds that of salt bridges and hydrogen bonds, as discussed for procathepsin L by Coulombe et al. (1996).

#### *Val44p and Asn48p strengthen P-domain binding to the enzyme*

As shown in Figure 4, outside of the core of the prodomain the hairpin between the  $\alpha$ 2p-helix and the  $\beta$ 1p-strand is stabilized by Val 44p interdigitating between Leu60p and Met62p. Furthermore, the Asn48p side chain is involved in two hydrogen bonds to main chain atoms of Leu60p, one of the residues anchored to the prosegment-binding loop of the enzyme. Val44p also forms van der Waals contacts to the side chain of Leu147 in the PBL. Alanine substitution of Val44p and Asn48p did not considerably shift the fluorescence emission to longer wavelengths (Fig. 8). The same mutations in the zymogen affected neither targeting nor maturation and secretion in transfected HEK cells (Kreusch et al. 2000). Although the P-domain structure of these mutants remained intact, the inhibitory capacity was shown to be reduced by about one order of magnitude (Fig. 8). The kinetics of complex formation of the propeptide mutants Val44 $\rightarrow$ Ala and Asn48 $\rightarrow$ Ala with the maternal enzyme revealed the reason for this: dissociation of the complex is much faster (0.02 sec<sup>-1</sup> and 0.03 sec<sup>-1</sup>, respectively) than

with other mutants and the wild-type propeptide (0.001 sec<sup>-1</sup>; all data from Table 1 in Schilling et al. 2001). Thus, these two residues, members of the ER(F/W)N(I/V)N motif, contribute to the tight binding of the P-domain to the enzyme by stabilization of the hairpin-anchor as well as by direct interaction with the harboring PBL.

#### *Autoprocessing may result from loss of the charge of Asp67p and Glu72p*

Ionic interaction of two highly conserved ionizable groups (Asp65p, Glu70p) in procathepsin L has been discussed to be involved in pH-dependent modulation of prosegment-enzyme interaction, i.e., autoprocessing (Coulombe et al. 1996). The homologous acidic residues in procathepsin S participate in the salt bridges Asp67p-Lys22p and Glu72p-Arg33p (Fig. 4; Table 1). The latter salt bridge connects the  $\alpha$ 3p helix to the N terminus of the  $\alpha$ 2p helix and may contribute to proper orientation of the  $\alpha$ 3 helix toward the active site cleft. The loss of the negative charge in position 67p was discussed to be involved in the pH-dependent activation of the papain precursor (Vernet et al. 1995). Substitution of each of these carboxylic residues by alanine (in separate experiments) did not considerably alter the structure and function of the cathepsin S propeptide (Fig. 8) (Schilling et al. 2001). These data suggest that dissociation of the proregion from the active-site cleft results from the loss of the negative charge of the two acidic residues, Asp 67p and Glu72p, by protonation. The functional consequence is that lowering the pH triggers the zymogen activation in a cooperative manner.

## Materials and methods

#### *Cloning and expression of the human procathepsin S (Cys25 $\rightarrow$ Ala) mutant in baculovirus-infected insect cells*

The complete sequence of human pre-procathepsin S, with the only potential glycosylation site in the proregion at Asn89p (HCS1/4) (Wiederanders et al. 1992), was cloned into the plasmid pcDNA3.1 (Invitrogen) as described previously (Kreusch et al. 2000). The Cys25 $\rightarrow$ Ala mutation, introduced to prevent autocatalytic processing of the zymogen, was created by PCR-based site-directed mutagenesis according to the ExSite protocol (Stratagene) with the following oligonucleotide primers (the altered bases are underlined):

sense 5'-TTGTGGTGCTGCCTGGGCTTTCAGTGCTGTGGG and antisense 5'-AAAGCCCAGGCAGCACCACAAGAACCTTGATAT TTCAC.

The mutant pre-procathepsin S gene was cloned into the vector pBlueBac4.5 (Invitrogen). The proenzyme DNA sequence was amplified using the primers 5'-TCACAGGATCCATGAAACG GCTGGTTTG (sense) and 5'-ACGGGGAAATTCCTAGATTTC TGGGTAAGAGG (antisense), including BamHI and EcoRI



restriction sites (*italic*); the sense primer also contained the triplet for the leading methionine (underlined). The insert was sequenced to check correct amplification and ligation. Recombinant baculovirus was generated by homologous recombination between the transfer vector and linearized Bac-N-Blue AcMNPV DNA in *Spodoptera frugiperda* 9 (Sf9) cells (Invitrogen). Insectin liposomes were used to optimize the transfection. Recombinant viruses were plaque-purified, amplified, and tested for their ability to express the human procathepsin S mutant. Large-scale expression of the recombinant protein was performed by G. Schmid (Hoffmann LaRoche, Basel, Switzerland). After centrifugation at 10,000 rpm, the supernatant of the culture medium was concentrated 10-fold, and stored in aliquots at  $-20^{\circ}\text{C}$  for subsequent purification.

#### Purification of recombinant human procathepsin S (Cys25→Ala) mutant

Capturing was performed with affinity chromatography using 50 mL concanavalin A sepharose (Amersham Pharmacia Biotech) packed in an XK 16/20 column (Amersham Pharmacia Biotech) at a flow rate of 2 mL/min. The gel was equilibrated with 0.1 M Tris (pH 7.4), 0.5 M NaCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , and 1 mM  $\text{MnCl}_2$ . After loading and washing (with three column volumes 0.1 M Tris at pH 7.4, 0.5 M NaCl), the protein was eluted with 0.25 M methyl- $\alpha$ -D-mannopyranoside, added to the washing buffer. Final purification of the recombinant human procathepsin S mutant to electrophoretic homogeneity was achieved by gel chromatography on a HiLoad Superdex-75 16/60 column (Amersham Pharmacia Biotech). Fractions containing procathepsin S were pooled and concentrated to 7.0 mg/mL by ultrafiltration (Amicon Centricon Plus-20, 5000 NMWL Millipore-Biomax 5).

#### Crystallization

The human procathepsin S Cys25→Ala mutant protein was crystallized using the hanging drop vapor diffusion method. Crystals were obtained by equilibrating 2  $\mu\text{L}$  of a 7.0 mg/mL protein solution with the same amount of reservoir solution (100 mM Tris-HCl, 200 mM magnesium acetate, 20% polyethylene glycol at final pH 7.5–7.8). Within 7 d at  $20^{\circ}\text{C}$ , platelet-shaped crystals appeared which grew to a maximum size of  $500 \times 250 \times 30 \mu\text{m}$  within 4 wk.

#### Collection of diffraction data

The crystals were flash-cooled at 100 K. No further cryoprotectant was needed in addition to the polyethylene glycol in the mother liquor. Initial data extending to 3 Å were collected using  $\text{CuK}\alpha$  radiation produced by an FR591 rotating anode generator (Nonius) and used for structure solution. The final data set at 2.1 Å resolution was subsequently collected using the Joint IMB Jena–University of Hamburg–EMBL synchrotron beamline X13 at Deutsches Elektronen-Synchrotron, Hamburg, at a wavelength of 0.803 Å. Diffraction intensities were recorded using a Mar Research CCD detector (X-ray Research). Data collection parameters are given in Table 2.

#### Data processing, structure solution, and refinement

Diffraction data were indexed, integrated, and scaled using the HKL package (Otwinowski and Minor 1997). Structure ampli-

**Table 2.** Data collection and refinement statistics

Space group	C 2 2 2 <sub>1</sub>
Unit cell parameters	$a = 59.8 \text{ \AA}$ , $b = 140.5 \text{ \AA}$ , $c = 78.6 \text{ \AA}$
Resolution range	30.0–2.10 Å
Number of observations	123,484
Number of unique reflections	19,350
$R_{\text{merge}}^{\text{a}}$	8.6% (47.1%)
$R_{\text{rim}}^{\text{b}}$	9.5% (53.6%)
$R_{\text{pim}}^{\text{c}}$	3.6% (24.1%)
$I/\sigma$	13.4 (3.0)
Completeness of data	97.9% (88.5%)
Refinement	
Resolution range	30.0–2.10 Å
Number of protein atoms	2459
Number of water molecules	242
Crystallographic $R$ -factor	19.8%
$R_{\text{free}}$	24.4%
RMSD (bonds)	0.009 Å
RMSD (angles)	1.35°
Average $B$ -factor (protein)	27.6 Å <sup>2</sup>

Values for the outermost resolution shell (2.14–2.10 Å) are shown in parentheses.

<sup>a</sup> $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - I(hkl)| / \sum_{hkl} \sum_i I_i(hkl)$ , where index  $hkl$  sums over all reflections and  $i$  sums over all equivalent and symmetry-related reflections (Stout and Jensen 1968).

<sup>b</sup> $R_{\text{rim}}$  is the redundancy-independent merging  $R$ -factor (Weiss and Hilgenfeld 1997), which is identical to  $R_{\text{meas}}$  of Diederichs and Karplus (1997).  $R_{\text{rim}} = \sum_{hkl} \{N(N-1)\}^{1/2} \sum_i |I_i(hkl) - I(hkl)| / \sum_{hkl} \sum_i I_i(hkl)$ , with  $N$  being the number of times a given reflection has been observed.

<sup>c</sup> $R_{\text{pim}}$  is the precision-indicating merging  $R$ -factor (Weiss and Hilgenfeld 1997).  $R_{\text{pim}} = \sum_{hkl} \{1/(N-1)\}^{1/2} \sum_i |I_i(hkl) - I(hkl)| / \sum_{hkl} \sum_i I_i(hkl)$ .

tudes were derived from the measured intensities by TRUNCATE (CCP4 1994). The asymmetric unit contains one molecule, corresponding to a packing density of  $2.31 \text{ \AA}^3/\text{Da}$  and a solvent content of  $\sim 47\%$  (Matthews 1968). Alignment of the procathepsin S amino acid sequence revealed the highest identity ( $\sim 55\%$ ) with procathepsin K (LaLonde et al. 1999; Sivaraman et al. 1999). Thus, procathepsin K (PDB entry code 1BY8) was used as the search model in molecular replacement calculations using the program AMoRe (Navaza 1994). The rotation and translation functions, calculated with reflections in the 15–4 Å resolution range, gave a solution with  $R = 0.461$  and a correlation coefficient of 0.418. The model was refined using CNS (Brunger et al. 1998). After rigid body refinement, simulated annealing, and energy minimization in torsion angle space,  $R_{\text{cryst}}$  at 3.0 Å could be reduced to 0.35. Synchrotron data were used for further refinement. Five percent of the unique reflections were set aside for the calculations of  $R_{\text{free}}$  (Brunger 1992). Several cycles of refinement, followed by refitting on a graphical workstation using the program O (Jones et al. 1991), resulted in a model that contained nearly all residues of procathepsin S (Fig. 1). Only residues 1p–2p and 93p–98p are missing due to poorly defined electron density. After introduction of 242 water molecules,  $R_{\text{cryst}}$  and  $R_{\text{free}}$  could be reduced to 0.198 and 0.244, respectively. Refinement statistics are given in Table 2. The atomic coordinates and structure factors have been deposited in the Protein Data Bank under entry 2COY.

### Chemical geometry and interactions

The chemical geometry of the structure was assessed by Ramachandran plots (Ramachandran and Sasisekharan 1968), calculated with the program PROCHECK (Laskowski et al. 1993). The secondary structure was defined using the program DSSP (Kabsch and Sander 1983). Hydrogen bonds, salt bridges, and van der Waals contacts were quantified with the program CONTACTS (CCP4 1994) and inspected with the program O (Jones et al. 1991). C–H $\cdots$  $\pi$  interactions were identified according to Brandl et al. (2001).

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