Catalysis of serine oligopeptidases is controlled by a gating filter mechanism

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Proteases have a variety of strategies for selecting substrates in order to prevent uncontrolled protein degradation. A recent crystal structure determination of prolyl oligopeptidase has suggested a way for substrate selection involving an unclosed seven-bladed β -propeller domain. We have engineered a disulfide bond between the first and seventh blades of the propeller, which resulted in the loss of enzymatic activity. These results provided direct evidence for a novel strategy of regulation in which oscillating propeller blades act as a gating filter during catalysis, letting small peptide substrates into the active site while excluding large proteins to prevent accidental proteolysis.

INTRODUCTION

The prolyl oligopeptidase family represents a relatively new group of serine peptidases, unrelated to the well known trypsin and subtilisin families, and includes enzymes of different specificities, such as prolyl oligopeptidase itself, dipeptidyl-peptidase IV, acylaminoacyl-peptidase and oligopeptidase B (Rawlings *et al.*, 1991; Rennex *et al.*, 1991; Polgár, 1994). They have a common feature to cleave substrates of no longer than ~30 amino acid residues in total. Prolyl oligopeptidase (EC 3.4.21.26) is involved in the metabolism of peptide hormones and neuropeptides (Wilk, 1983; Mentlein, 1988; Cunningham and O'Connor, 1997), and has recently gained pharmaceutical interest since specific inhibitors can relieve scopolamine-induced amnesia (Yoshimoto *et al.*, 1987; Attack *et al.*, 1991; Miura *et al.*, 1995; Portevin *et al.*, 1996). The enzyme activity has also been associated with depression (Maes *et al.*, 1994; Williams *et al.*, 1999) and blood pressure regulation (Welches *et al.*, 1993).

Our crystal structure determination revealed that the enzyme contains an N-terminal peptidase domain with an α/β hydrolase fold, and its catalytic triad (Ser554, His680, Asp641) is covered by the central tunnel of an unusual β -propeller (Fülöp *et al.*, 1998). The β -propeller fold has been found in many different structures with diverse biological functions (for a recent review see Fülöp and Jones, 1999). Most of them use their central tunnel, or the entrance to that tunnel, to coordinate a ligand or to carry out some catalytic function that has to be preserved by the structural rigidity of the propellers. The four-stranded antiparallel β -sheets are radially arranged around a central tunnel and they pack face to face. The circular structure is stabilized by a molecular 'Velcro', a network of hydrogen bonds between neighbouring strands of the last blade, which is formed by both termini of the propeller domain. Interestingly, the smaller four-bladed haemopexin propellers developed another variant, with a disulfide bond between the first and last blades (Paoli *et al.*, 1999). Unlike in all the other β -propellers (Fülöp and Jones, 1999), the 'Velcro' is not closed in prolyl oligopeptidase by any of these ways, there are only hydrophobic interactions between its first and seventh blades (Fülöp *et al.*, 1998). The narrow entrance of the propeller (~4 Å) opposite to the catalytic domain is much smaller than the diameter of an average peptide (6–12 Å), but it could be enlarged by partial separation of the unclosed blades 1 and 7. This mechanism could provide an access to the active site for oligopeptides, and at the same time would protect larger, structured peptides and proteins from accidental proteolysis in the cytosol, where the enzyme is primarily localized. In order to provide insights for the substrate selection mechanism, we have covalently connected the first and seventh blades of the propeller domain by a disulfide bond, and subsequently determined the crystal structure and examined the catalytic properties of this variant.

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RESULTS AND DISCUSSION

Engineering the disulfide bond

The crystal structure of prolyl oligopeptidase indicated that residue 397 of blade 7 is in a favourable position to form a disulfide bridge with Cys78 of blade 1. Residue 397, which is glutamine in the wild-type enzyme, was changed to cysteine by site-specific mutagenesis, and the Q397C variant was cloned, expressed and purified similarly to the wild-type enzyme (Szeltner *et al.*, 2000a). The amino acid substitution did not result in a significant alteration in the enzyme activity, indicating that either the separation of the blades was not essential for catalysis or the disulfide bond had not been formed during protein folding and purification. However, the activity decreased during storage, suggesting that the disulfide bond formation is a slow process and could be affected with the treatment of a mild oxidizing agent, e.g. oxidized glutathione.

Porcine prolyl oligopeptidase has 16 free cysteine residues. Therefore, in addition to the thiol groups involved in disulfide bond formation between residues 78 and 397, the effects of oxidized glutathione on other cysteines should also be considered. Indeed, mammalian prolyl oligopeptidases can be inhibited with thiol reagents, such as iodoacetamide, *N*-ethylmaleimide or *p*-chloromercuribenzoate (Polgár, 1991; Szeltner *et al.*, 2000b). Crystal structure determination has revealed that Cys255 is found in the active site region, thus it could be a candidate for interfering with catalysis when it is blocked (Fülöp *et al.*, 1998). On the other hand, prolyl oligopeptidase from *Flavobacterium meningosepticum* (Yoshimoto *et al.*, 1991; Chevalier *et al.*, 1992) has a threonine residue at position 255 and cannot be inhibited with thiol reagents. To eliminate the adverse effects of Cys255 during the disulfide bond formation, we have prepared the double mutant C255T/Q397C. Again an active enzyme was obtained, which formed the expected disulfide bond upon oxidation with oxidized glutathione.

Table I. Inactivation of prolyl oligopeptidase

Enzyme ^a	pH (±0.05)	k_{inact} (h ⁻¹) Oxidized glutathione	
		1 mM	3 mM
Wild type	8.0	0.24 ± 0.02	
C255T	8.0	0.039 ± 0.003	
C255T	9.0	1.83 ± 0.19	
Q397C	8.0	0.43 ± 0.02	
C78A/C255T	8.0	0.044 ± 0.005	
C78A/C255T	9.0	0.84 ± 0.03	
C255T/Q397C	8.0	0.118 ± 0.004	0.25 ± 0.02
C255T/Q397C	9.0	4.0 ± 0.3	10.9 ± 0.7
C255T/Q397C ^b	9.0	0.099 ± 0.006	

^aThe reactions were measured at 28°C.

^bWithout oxidized glutathione.

Fig. 1. Oxidation of prolyl oligopeptidase variants. Reaction of the enzymes was carried out with 1.0 mM oxidized glutathione at pH 9.0 and 28°C. Open circles, C78A/C255T variant; filled circles, C255T/Q397C variant. The curves represent single exponential decays.

Kinetics of the disulfide bond formation

Table I shows the changes in the inactivation rate constants of the different variants of prolyl oligopeptidase during incubation with 1 mM oxidized glutathione. Examples of change in activity are shown in Figure 1. While the wild-type enzyme is considerably inhibited, the rate constant for inactivation of the C255T mutant is smaller by one order of magnitude. The strongly reduced inhibition rate confirms that out of the 16 cysteines, Cys255 predominantly accounts for the inhibition of the wild-type prolyl oligopeptidase. Inhibition of the wild-type enzyme is incomplete as found earlier with iodoacetamide and other thiol reactants (Polgár, 1991; Szeltner *et al.*, 2000b). This indicates that Cys255 is not directly involved in catalysis, and its blocking sterically hinders the substrate from proper binding. In the absence of oxidized glutathione, both the wild-type enzyme and its C255T variant practically retain their activity during a 1 day incubation period (not shown).

The C255T/Q397C variant is inhibited with the oxidized glutathione somewhat more slowly than the wild-type enzyme (Table I). This rate must predominantly be due to the formation of a disulfide bond between residues 78 and 397, and not to the reaction of other cysteines of prolyl oligopeptidase. This follows from the control experiment indicating that the C255T variant, which is incapable of forming a disulfide bond with glutathione, but bears all the potentially reactive other cysteines, is inactivated much more slowly than the C255T/Q397C variant. In contrast to the wild-type enzyme, the C255T/Q397C mutant is virtually completely inactivated. Unfortunately the reaction is rather slow and it is difficult to follow to its end, therefore it cannot be excluded that the enzyme with a fastened β -propeller still retains some activity. The Q397C variant, where both disulfide bond formation and Cys255 blocking can occur, is inactivated most rapidly.

It should be noted that the C255T variant may not be a perfect control for the C255T/Q397C variant because Cys78 could also react with the oxidized glutathione, and the bulky label may obstruct the substrate access between blades 1 and 7. The disulfide bond formation between the two blades must proceed with the same mechanism, involving the reaction of the oxidized glutathione with Cys78 or Cys397 as the first step. The second

step is the displacement of the glutathione from the intermediate disulfide by the neighbouring free thiol group. Disulfide bond formation between Cys78 of the wild-type enzyme and a thiol compound has already been shown (Fülöp *et al.*, 1998). We have prepared the C78A/C255T variant of prolyl oligopeptidase to estimate the contribution of oxidation of Cys78 to the enzyme inactivation. Data in Table I show that the inactivation rate of this variant is considerably smaller at pH 9.0 than that of the C255T variant where Cys78 is present. It appears that besides Cys255, Cys78 could be another important residue in the inactivation of prolyl oligopeptidase by oxidized glutathione. Despite the presence of 14 other thiol groups in the C255T variant of prolyl oligopeptidase, Cys78 accounts for >50% of the inactivation at pH 9.0 (Table I). These results also show that ~80% of inactivation originates from the disulfide bond formation.

The low rate of disulfide bond formation at pH 8.0 can readily be explained in terms of the high pK_a values of Cys78 and Cys397, owing to their hydrophobic environment. Indeed, we have found that the pK_a of Cys255, which is located in a partially hydrophobic environment, is as high as 9.77 (Szeltner *et al.*, 2000b). The nucleophilicity of an -SH group is negligible compared with its ionized form. Thus, if the pH of the reaction is far below the pK_a of the thiol group, an increase in pH by 1 unit will provide a 10-fold higher rate constant. At pH 7.0 the disulfide bond formation was so slow that we could not determine its rate constant. Increasing the pH from 8.0 to 9.0, however, resulted in >10-fold rate enhancement (Table I). This may be associated with the increased flexibility of the enzyme at high pH (Polgár, 1995). A similar pH effect was observed with the C255T and C78A/C255T variants. When either of these rate constants had been subtracted from that of the C255T/Q397C variant, the corrected rate enhancements were still >10-fold.

No appreciable damage occurs during oxidation of the C255T/Q397C variant, as an overnight incubation with 10 mM dithioerythritol recovers the enzyme activity to at least 90%. In the absence of oxidized glutathione, the activity of the enzyme is practically retained during a 1 day incubation at pH 8.0 and 28°C, indicating that the oxygen dissolved in the medium is not effective enough to promote the disulfide bond formation. However, when the pH is raised to 9.0, a slow inactivation takes place even in the absence of oxidized glutathione (Table I), which reverted in the presence of dithioerythritol.

Structure of the C255T/Q397C mutant

Figures 2 and 3 show the disulfide formation between residues 78 and 397. The covalent bond between the first strand (innermost) of the first blade and the second strand of the seventh blade of the propeller domain did not induce any conformational change; the temperature factor distribution is similar to the wild-type enzyme. The high quality of the electron density map at this region suggests that the level of disulfide formation in the crystal is complete. The C255T mutation is also clearly visible in the electron density map (not shown) without causing any further perturbation in the structure.

While the three-dimensional structure is not affected by the engineered disulfide bridge, the protein stability significantly increased. Differential scanning calorimetry measurements with the wild-type enzyme revealed two well separated peaks, which corresponded to the denaturation of the two domains

Fig. 2. Electron density around the disulfide bond of the C255T/Q397C variant of prolyl oligopeptidase. The SIGMAA (Read, 1986) weighted $2mF_o - DF_c$ electron density using phases from the final model is contoured at 1σ level, where σ represents the r.m.s. electron density for the unit cell. Contours $>1.4 \text{ \AA}$ from any of the displayed atoms have been removed for clarity. The position of the Glu397 side chain of the wild-type enzyme is also shown in thin lines. The picture was drawn with MolScript (Kraulis, 1991; Esnouf, 1997).

(Z. Szeltner, unpublished data). The peak observed at the lower temperature ($T_m = 44.4^\circ\text{C}$), and very likely representing the denaturation of the propeller domain, disappeared in the enzyme variant containing the disulfide bridge, and merged into the second peak ($T_m = 53.8^\circ\text{C}$), indicating the enhanced stability of the propeller bearing the disulfide bond.

The experiments reported here have clearly proved the unique feature of the β -propeller domain of prolyl oligopeptidase, where its blades oscillate between open and closed forms during catalysis. The lack of the molecular 'Velcro' does not alter the symmetrical structure of the propeller domain of prolyl oligopeptidase, but makes it flexible for the required regulatory function. The propeller opening between blades 1 and 7 for substrate access is also supported by the finding that blocking of Cys78 causes a partial inhibition. The limited accessibility of Cys78 by a hexapeptide, i.e. oxidized glutathione, suggests that the substrate approaches the active site through the pore at the propeller end. The propeller opening between its first and seventh blades also increases the pore diameter at the bottom of the propeller providing an entrance for substrates to the active site.

METHODS

Protein expression and purification. Prolyl oligopeptidase of porcine brain was expressed in *Escherichia coli* and purified as described (Szeltner *et al.*, 2000a). The Q397C mutation was introduced into the pTrc/PO/C255T plasmid with the two-step procedure as described for the Y473F mutant (Szeltner *et al.*, 2000a). The following primers were used: 5'-ACGGAAATaTTC-TATgtTTTACTTCC-3' and 3'-TGCCTTtAaAGATAacaAAATG-AAGG-5'. An extra recognition site for *SspI* restriction enzyme

Fig. 3. Ribbon diagram of the C255T/Q397C variant of prolyl oligopeptidase. **(A)** The N-terminal (residues 1–78) and C-terminal (residues 428–710) parts of the catalytic domain are coloured in dark blue and red, respectively. The regulatory β -propeller domain, residues 73–427, is colour-ramped from blue to orange. The catalytic residues (Ser554, His680, Asp641) are shown in a ball-and-stick representation. The disulfide bond connecting blades 1 and 7 is shown in red. **(B)** The protein chain of the β -propeller domain only, coloured as in (A), and viewed perpendicular to that down the pseudo-7-fold axis. Residues (Lys82, Glu134, His180, Asp242, Lys389 and Lys390) narrowing the entrance to the tunnel of the propeller are shown in a ball-and-stick representation. Drawn with MolScript and rendered with Raster3D (Merritt and Murphy, 1994).

Table II. Data collection and refinement statistics

Data collection	
resolution ($\text{\AA}$)	21–1.7
observations	281 721
unique reflections	82 125
R_{sym}^a	0.061
completeness (%)	94.6
Refinement	
non-hydrogen atoms	6618 (including 866 waters)
$R_{\text{cryst}} (2\sigma \text{ cutoff})^b$	0.179
reflections used	77 351
$R_{\text{free}} (2\sigma \text{ cutoff})^c$	0.206
reflections used	3279
R_{cryst}^b	0.184
reflections used	78 797
R_{free}^c	0.210
reflections used	3328
$R_{\text{cryst}} (\text{all data})^b$	0.186
mean temperature factor ($\text{\AA}^2$)	15.7
R.m.s.ds from ideal values	
bonds ($\text{\AA}$)	0.008
angles ($^\circ$)	1.5
mean coordinate error ($\text{\AA}$) ^d	0.14

^a $R_{\text{sym}} = \sum_j \sum_h |I_{h,j} - \langle I_h \rangle| / \sum_j \sum_h \langle I_h \rangle$ where $I_{h,j}$ is the j th observation of reflection h , and $\langle I_h \rangle$ is the mean intensity of that reflection.

^b $R_{\text{cryst}} = \sum |F_{\text{obs}}| - |F_{\text{calc}}| / \sum |F_{\text{obs}}|$ where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively.

^c R_{free} is equivalent to R_{cryst} for a 4% subset of reflections not used in the refinement (Brünger, 1992b).

^dDetermined by the SIGMAA method (Read, 1986).

of the pTrc99A vector. This *Bam*HI fragment containing the Q397C mutation was excised and inserted into the place of the corresponding fragment of the wild-type pTrc/PO vector. The fragments were isolated with agarose gel electrophoresis and purified from the gel with QIAquick Gel Extraction Kit (Qiagen). The ligation mixture was transformed into *E. coli* DH5 α , and the DNA was isolated from the colonies grown on LB. The correct orientation of the inserted fragment was checked with restriction mapping. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs.

The C78A mutation and a new *Dra*I restriction site were introduced into the wild-type pTrc/PO plasmid with the oligonucleotide primers 5'-CCCAAGTATAGTgcCCACTTAAAA-AGG-3' and 3'-GGGTTCATATCAcggGTGAAaTTTTTCC-5'. Then the *Eco*II-*Bsm*I fragment was cleaved from the resulting plasmid and inserted into the corresponding *Eco*II-*Bsm*I site of the pTrc/PO/C255T vector. The new plasmid was used for the preparation of the C78A/C255T enzyme variant.

Kinetics. The reaction of prolyl oligopeptidase with Z-Gly-Pro-Nap (Bachem Ltd.) was measured fluorometrically (Polgár, 1994). Inactivation of prolyl oligopeptidase and its variants was

(underlined) was also created with silent mutation to check the incorporation of the mutagenic oligonucleotides into the PCR product. The Q397C single mutant was prepared from the C255T/Q397C double mutant. Two *Bam*HI sites are found in plasmid pTrc/PO/C255T/Q397C, one at nucleotide 958 of the prolyl oligopeptidase gene, and the other at the polycloning site

performed in 1.0 ml of reaction mixture containing ~400 nM enzyme and 1.0 or 3.0 mM oxidized glutathione. Aliquots (10–40 µl) were withdrawn at appropriate times, and the remaining activities were measured as described above. Dithioerythritol (used for storage) was removed by gel filtration on a Sephadex G-50 column before enzyme oxidation. Buffers were purged with nitrogen before use.

Crystallization, X-ray data collection and structure refinement. The C255T/Q397C variant of prolyl oligopeptidase was crystallized the same way as the wild-type enzyme (Fülöp *et al.*, 1998). Crystals belong to the orthorhombic space group $P2_12_12_1$ with cell dimensions $a = 70.7$, $b = 99.7$ and $c = 110.9$ Å. X-ray diffraction data were collected at 100 K on a MAR345 image plate detector at the beam line X11, EMBL, Hamburg using 0.909 Å wavelength. Data were processed using the HKL suite of programs (Otwinowski and Minor, 1997). Refinement of the structure was carried out by alternate cycles of X-PLOR (Brünger, 1992a) and manual refitting using O (Jones *et al.*, 1991). The refinement was based on the 1.4 Å resolution model of wild-type enzyme (Fülöp *et al.*, 1998) (Protein Data Bank code: 1qfm). A bulk solvent correction allowed all measured data to be used. Water molecules were added to the atomic model at the positions of large positive peaks ($>3.0\sigma$) in the difference electron density, only at places where the resulting water molecule fell into an appropriate hydrogen-bonding environment. Restrained isotropic temperature factor refinements were carried out for each individual atom. The final model contains all the 710 amino acid residues, nine glycerol and 866 water molecules. Statistics for the data processing and refinement are given in Table II. Coordinates have been deposited in the Protein Data Bank, code 1e5t.

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