STRUCTURE AND FUNCTION OF OLIGOPEPTIDASES: CATALYSIS IN THE ENZYME FAMILY S9A

Synopsis of Ph.D. Thesis

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Introduction

Subjects of this thesis are prolyl oligopeptidase (POP) and oligopeptidase B (OPB). POP is implicated in a variety of disorders of the central nervous system. Inhibitors to the enzyme are described as cognitive enhancers. OPB promotes infection by trypanosomes. Inhibitors of the enzyme hinder the invasion of parasites into the host cells.

POP and OPB are members of a relatively new serine protease family, the prolyl oligopeptidase family (subfamily S9A). The enzymes of this family contain two domains: a catalytic peptidase domain structurally related to lipases of the α/β-hydrolase fold rather than to the classic serine peptidases (e. g. trypsin) and a regulator β-propeller domain. The peptidase domain of POP is built up of residues 1-72 and 428-710 and the residues between these two portions constitute the propeller domain. The seven-bladed propeller of POP has an open *velcro* topology: the propeller is stabilized only by hydrophobic interactions between the first and last blades.

The propeller covers the active site located in a large cavity at the interface of the two domains thereby it accounts for excluding large, structured peptides and selecting smaller oligopeptides so for the oligopeptidase activity. However, the pathway for the substrate to the active site is not cleared by the rigid crystal structure, which displays an apparently firm two-domain construction, which should exclude even the smallest peptide. So substrate access and efficient activity require rapid conformational changes that open up the protein structure. The narrow entrance of the propeller is much smaller than the diameter of an average peptide, but it could be enlarged by the partial separation of the unclosed blades 1 and 7. A regulatory mechanism involving oscillating propeller blades required for substrate entry was supported by engineering a disulfide bridge between blades 1 and 7, which inactivated the porcine POP enzyme. Substrate approach between the two domains was suggested for the more compact *Pyrococcus furiosus* (*Pfu*) POP that is capable of hydrolyzing proteins: the enlarging the crevice between the two domains by moving apart might enable even larger substrates to enter the active site.

In the absence of crystal structure, kinetic measurements provided information about the action of oligopeptidase B. It is known, that the enzyme cleaves after lysine or arginine residues and hydrolyzes much faster peptides with two adjacent basic residues

than those with a single base. Studies with small synthetic substrates revealed similarities to the POP: complex pH-rate profiles, sensitivity to ionic strength and rate-limiting substrate-induced conformational change were demonstrated for both enzymes.

Aims

This research aims to reveal the nature of the oligopeptidase activity as well as the contribution of the functionally important groups to the catalysis.

1. The catalytic triad (Ser, His, Asp) is of primary importance in the catalysis of serine proteases. The competence of the catalytic Asp and the operation of the triad have been the subject of a long discussion. We aim to establish the contribution of the active site D641 of porcine POP to the catalysis. To this end we examine the D641N and D641A variants.
2. A further important part of the catalytic machinery of serine peptidases is the oxyanion binding site. According to the homology with POP, the oxyanion binding site of the OPB from *E. coli* contains the Tyr452 the catalytic contribution of which is investigated with the Tyr452Phe variant.
3. Substrate specificity is determined by enzymatic substrate binding sites. Substrate recognition properties and the role of the subsites S1 and S2 in the catalysis of OPB from *E. coli* are studied using oligopeptide substrates.
4. It is a peculiarity in the catalysis of oligopeptidases that the propeller acts as a gating filter. However, the mechanism of substrate selection and the route for the substrate to the catalytic groups are not known. In order to reveal the pathway and mechanism in the case of POP, we investigate the possibilities of substrate access through the propeller and between the domains.
5. Some members of the prolyl oligopeptidase family are believed to hydrolyze larger peptides and proteins. These enzymes are not rigorously oligopeptidases. The POP from *Pyrococcus furiosus (Pfu)* is reported to be capable of acting on protein substrates as well. The study of the thermophilic enzyme is expected to reveal the background for the activity unusual in the family.

Methods

- Enzyme preparation: recombinant protein expression in *E. coli*, protein purification, site-specific mutagenesis
- Enzyme kinetics: activity assay, substrate binding, enzyme inhibition, determination of rate constants, kinetic and activation parameters for enzyme reactions
- Protein stability measurements – denaturation and refolding
- Chemical modification of proteins: alkylation, disulfide bond formation
- Techniques: PCR; chromatography, gelelectrophoresis, differential scanning calorimetry; ultraviolet-visible, fluorescence and circular dichroism spectroscopy

Results and conclusion

1. The catalytic triad of the prolyl oligopeptidase

- The activities of the D641N and D641A variants were similarly reduced compared with the wild type POP: it is the negative charge of the aspartate that is important to the catalysis.
- Both variants displayed virtually no change in k_{cat}/K_m with an ester substrate, but two, three and six orders of magnitude reduction in k_{cat}/K_m was measured for a thiolester, amide and octapeptide substrate, respectively: the active site lacking the D641 is less capable of hydolyzing stronger bonds. Such a dependence of the activity on the substrate leaving group was not the case with trypsin.
- The reduced activity of the mutants held over the entire pH-range, in contrast to trypsin, by which the reduction at alkaline pHs was much less pronounced.
- The correct orientation of the catalytically competent tautomer form of His680 of the catalytic triad was found in the crystal structure of both variants, in contrast to the incorrect tautomer of the D102N trypsin variant.

2. The oxyanion binding site of the oligopeptidase B from *E. coli*

- The Y452F variant displayed virtually no change in k_{cat}/K_m with ester substrates, but approximately two orders of magnitude reduction in k_{cat}/K_m was measured for most amide and peptide substrates. For a small, less specific amide substrate the reduction

was three orders of magnitude. The catalytic contribution of the OH group of Tyr452 depends on the substrate.

- Using the Y452F variant non-productive binding was observed with a small, less specific ester substrate. The oxyanion binding site is not only an effective electrophilic catalyst but it is also a binding entity that accounts for abolishing potential non-productive binding.

3. The substrate binding of the oligopeptidase B from *E. coli*

- The OPB from *E. coli* is very sensitive to ionic strength, which suggests that the arginines of the substrate interact with the carboxylate ions of the enzyme. The dependence of the activity on salt concentration is different with substrates bearing one and two arginine residues.
- On the basis of a 3D model, two carboxyl dyads Glu576, Glu578 and Asp460, Asp462 can be assigned as binding sites for the arginines P1 and P2, respectively.
- The carboxyl dyads of the binding sites are also involved in substrate inhibition at high substrate concentrations and in the enzyme activation by $CaCl_2$. The ionization of one of the subsite carboxyls affects the pH-rate profile as well.

4. The mechanism of substrate selection

- Introducing disulfide bridges to the expected substrate entry sites, the oscillating motions required to the activity may be eliminated. The disulfide bond between the propeller blades 1 and 7, distant from the peptidase domain could not formed. The disulfide bond between the blades 1 and 7, close to the peptidase domain was produced, which resulted in the decrease of the activity. The disulfide bond between the domains was easily created and inactivated the enzyme. Cross-linking the domains impeded binding of an octapeptide substrate. These results indicate that flexibility of the propeller is essential for the activity of POP. Separation of the domains is also required not only for the activity but also for the substrate binding.
- The unclosed seven-bladed propeller of the porcine POP can be expressed separately from the peptidase domain. Shortened, six-bladed propeller variants can also be prepared. Our results suggest that unclosed β-propellers are autonomous entities, which can be prepared artifically

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- The separated propeller of POP is fairly stable: it is more stable than the parent enzyme. This suggests that the structure of the separated propeller is destabilized by coupling with the peptidase domain. The enhanced stability of the propeller indicates that it cannot open up to permit the substrate entry through this domain. However, the flexibility of the propeller linked to the protease domain could facilitate the concerted movements necessary for catalysis.
- *In silico* methods performed in collaboration identified a smaller tunnel at the inter-domain region as the only potential pathway for the substrate to the active site. The N-terminal segment (10-40 residues) is part of the tunnel. The shortened (Δ55 and Δ71) variants of the porcine POP could not been expressed in soluble form so that the role of the N-terminal fragment could not been investigated. In contrast, the Δ32 variant of *Pfu* POP could be prepared. While the activity, kinetic and activation parameters of the shortened variant differed only slightly from those of the native enzyme, the stability of the protein was damaged substantially compared to the remarkably stability of the thermophilic wild-type enzyme. Our results suggest that the tunnel-forming N-terminal segment is also important for the stabilization of the proper protein structure.
- Other part of the entry tunnel constitutes a flexible loop of the propeller (residues 192-205). A nicked enzyme was prepared by cleaving the loop by limited proteolysis. The rate constant for the reaction with Z-Gly-Pro-Nap was remarkably higher with the nicked enzyme than that with the wild-type enzyme. The kinetic and activation parameters for substrate binding were also different between the native and the cleaved enzymes. The observed functional importance of the tunnel-forming loop in substrate binding supports the involvement of this loop in substrate passage.

5. Kinetic study of the POP from *Pyrococcus furiosus*

Detailed kinetic investigation of the catalytic properties of the *Pfu* POP revealed many differences to a study reported previously. We have demostrated that

- the *Pfu* POP does not display appreciable activity against azocasein: its efficacy to cleave this protein substrate is ~2500 weaker than that of the trypsin. This indicates that the *Pfu* POP cannot hydrolyze peptides regardless of size. It is rather an oligopeptidase restricted to oligopeptide substrates.
- the reported autolysis was an artefact: after longer incubation of *Pfu* POP at high temperature, a minor cleavage could only be observed, which cannot be valued a usual

enzymatic reaction. The open form of the enzyme is only able to accommodate large peptides. However, in the open form the catalytic triad is distorted so that the open form cannot be active. Hence, the hydrolysis of proteins may only occur with very low efficacy under extreme conditions, as observed with the *Pfu* POP.

Summary

We have studied two serine peptidases of the prolyl oligopeptidase family (subfamily S9A). The main results are as follows:

- Substrate-dependent competency of the catalytic triad was demonstrated for the prolyl oligopeptidase. It was found that the triad operates as a charge stabilizing system.
- The catalytic effects of the oxyanion binding site of the oligopeptidase B were shown to depend on the nature of the substrate. As a peculiarity of the action of OPB, it was found that the oxyanion binding site is not just an electrophilic catalyst and the carboxyl dyads of the subsites S1 and S2 are not just substrate binding sites but each individual group plays a multifunctional role in the catalysis.
- We have demonstrated that the propeller has stable structure, indicating that it cannot open up and the substrate should approach the catalytic site through a tunnel between the peptidase and the propeller domains. This requires the concerted movements of the two domains.

Publications serving as a basis of the dissertation:

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3. Szeltner Z, Rea D, Juhasz T, Renner V, Fulop V and Polgar L „Concerted structural changes in the peptidase and the propeller domains of prolyl oligopeptidase are required for substrate binding“ *J Mol Biol* **340** (2004) 627-37
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