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The loops facing the active site of prolyl oligopeptidase are crucial components in substrate gating and specificity

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ABSTRACT

Prolyl oligopeptidase (POP) has emerged as a drug target for neurological diseases. A flexible loop structure comprising loop A (res. 189–209) and loop B (res. 577–608) at the domain interface is implicated in substrate entry to the active site. Here we determined kinetic and structural properties of POP with mutations in loop A, loop B, and in two additional flexible loops (the catalytic His loop, propeller Asp/Glu loop). POP lacking loop A proved to be an inefficient enzyme, as did POP with a mutation in loop B (T590C). Both variants displayed an altered substrate preference profile, with reduced ligand binding capacity. Conversely, the T202C mutation increased the flexibility of loop A, enhancing the catalytic efficiency beyond that of the native enzyme. The T590C mutation in loop B increased the preference for shorter peptides, indicating a role in substrate gating. Loop A and the His loop are disordered in the H680A mutant crystal structure, as seen in previous bacterial POP structures, implying coordinated structural dynamics of these loops. Unlike native POP, variants with a malfunctioning loop A were not inhibited by a 17-mer peptide that may bind non-productively to an exosite involving loop A. Biophysical studies suggest a predominantly closed resting state for POP with higher flexibility at the physiological temperature. The flexible loop A, loop B and His loop system at the active site is the main regulator of substrate gating and specificity and represents a new inhibitor target.

1. Introduction

Prolyl oligopeptidase (POP, PREP, PEP) (EC 3.4.21.26) is a member of a family of serine peptidases unrelated to the classic trypsin and subtilisin. The family includes enzymes of different specificities, like POP itself, dipeptidyl peptidase IV, acylaminoacyl peptidase, and oligopeptidase B [1]. POP is a cytosolic enzyme that selectively cleaves oligopeptides not greater than ~30 amino acids in length. POP is implicated in the metabolism of peptide hormones and neuropeptides [2–4] although some of the previously identified neuropeptide substrates do not appear to be hydrolyzed in vivo upon closer inspection [5]. Specific inhibitors have been shown to relieve scopolamine-induced amnesia [6], to ameliorate memory loss caused by age, brain lesions, or amnesic drugs [7,8] and were neuroprotective under various conditions [9–11], resulting in significant pharmaceutical and academic interest. It was reported that POP has roles in the phosphoinositide cycle [12–14], intracellular transport [15], cellular differentiation [16], inflammation [17], angiogenesis [18], and Parkinson's disease [19]. Involvement in some processes may involve non-catalytic protein–protein interactions [14,20]. POP is emerging as an important drug target in neurological diseases.

The crystal structure of POP [21] showed that the enzyme is composed of a catalytic domain with an α/β-hydrolase fold and a 7-bladed β-propeller domain. The β-propeller covers the active site that is situated in a central cavity at the domain interface. Crystal structures of mammalian POP-ligand complexes show the ligand completely buried in the interior of the closed enzyme, and therefore do not reveal how substrates and products access and exit the active site. Clearly, conformational changes are involved, as was demonstrated earlier when kinetic isotope effects showed that the rate-determining step of catalysis is a physical rather than a chemical

Abbreviations: Abz, 2-aminobenzoyl; BNA, β-naphthylamide; CD, circular dichroism; DLS, dynamic light scattering; DSC, differential scanning calorimetry; DTT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; EDDnp, N-(2,4-dinitrophenyl)-ethylenediamine; MES, 4-morpholineethanesulfonic acid; MD, molecular dynamics; Ni-NTA, nickel-nitrilotriacetic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PCR, polymerase chain reaction; POP, prolyl oligopeptidase; Phe(NO_2), p-nitrophenylalanine; res., residue; SEC, size-exclusion chromatography; TEV, tobacco etch virus; T_m, melting temperature

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step [22]. It was previously suggested that substrates could be gated between the first and last propeller blades, allowing only short peptides into the active site via the propeller central tunnel [23]. However, the propeller domain of POP was found to be too rigid for such a regulatory function [24]. Limiting the flexibility between the propeller and catalytic domains with an engineered disulfide bridge inactivated the enzyme, suggesting a requirement for some inter-domain motion [25]. Flexibility of the loop structure at the domain interface was suggested to be required for efficient catalysis [25,26]. This loop structure comprises a loop of the propeller domain (loop A, res. 189–209), a facing loop of the peptidase domain (loop B, res. 577–608), and other catalytically important loops as seen in Fig. 1. In contrast to all current mammalian POP crystal structures, the *Sphingomonas capsulata* [27] and later *Aeromonas punctata* [28] POP crystal structures revealed the enzyme in an open state, in which the peptidase and propeller domains are widely separated about a hinge region. This suggested that the substrate approaches the active site between the two domains. The inhibitor Z-pro-prolinal was soaked into the *A. punctata* crystals, and loose crystal packing resulted in ligand binding and consequent domain closure, resulting in the closed state, ligand-bound crystal structure. The authors proposed that *S. capsulata* and *A. punctata* POP reside in closed and open states, respectively, and substrate causes domain separation and closure, respectively, supporting an induced fit mechanism. Despite the bacterial POP crystal structures, it is still unknown whether bacterial or mammalian POP in solution is in an open or closed resting state or in equilibrium between the two states. Crystal structures of ligand-free porcine POP in the closed state demonstrate that ligand binding is not required for domain closure, and that at least some of the enzyme molecules reside in the resting closed state, supporting a conformational selection mechanism. However, it is also possible that the enzyme remains closed during catalysis, requiring only movements of loop A and/or other loops and only limited inter-domain movements to allow substrate entry, as was suggested by earlier MD simulation studies [26] and by a recent study [29]. If this is the case, the open state observed in the bacterial crystal structures may be an artifact of the very high local protein concentration in the crystal. Alternatively, domain opening may be exclusive to, or more prevalent in, the substrate gating of the bacterial POPs.

The main objectives of this work were: 1) to test experimentally how surface loops modulate ligand binding and catalysis using mutagenesis, enzyme kinetic studies and X-ray crystallography. In this way, we checked the validity of former computer simulation studies that suggested that this loop system is the site of ligand entry. 2) to investigate the structural dynamics of POP and to reveal the contribution made by the flexible loops and ligand binding, and 3) to investigate the resting state of POP in solution and to probe the nature of the various POP forms observed in native gels.

Supported by mutagenesis, crystallography and biophysical studies, this work demonstrates that the flexible loop system involving loop A, loop B, the loop holding the catalytic His (His loop) and the N-terminus of POP are important elements of the substrate gating mechanism and are required for the fine control of the catalysis.

2. Materials and methods

2.1. Protein engineering and mutagenesis

The expression and purification of the variants mutated in loop A and loop B (T202C and T202C/T590C, respectively) and of the variant lacking loop A are described in Ref. [29]. Novel mutants (T204A, H680A and D149A) were created using complementary mutagenic primers, the wild-type POP bearing pSKPOP plasmid as template and the Quick Change Lightning PCR-based mutagenesis kit and protocol (Stratagene). The mutated proteins were expressed in a Rosetta *Escherichia coli* strain and purified as described for the wild-type POP elsewhere [30].

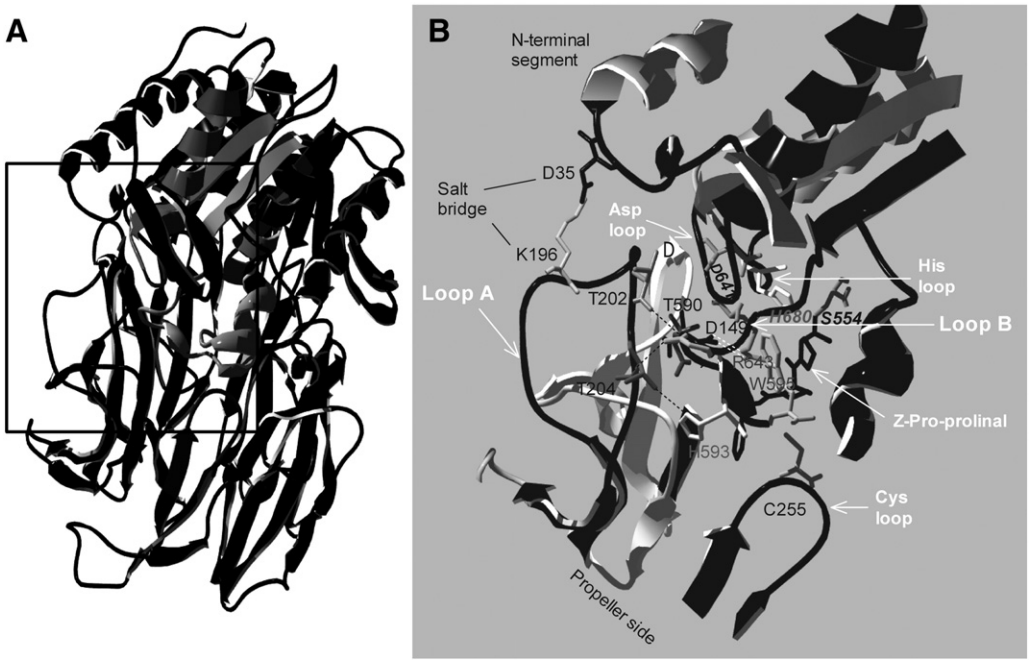

Fig. 1. Schematic representation of the structure of POP. (A) The overall structure of POP. (B) Part of POP, comprising the flexible loop structure. Loop A (res. 189–209) of the propeller, loop B (res. 577–608) of the catalytic domain and the loops holding D641, His680 and Cys255 are shown in black. The part of the N-terminus holding D35 are also shown as well as the loop that holds D149 (in white and signed with D). The mutated residues, the catalytic amino acids, the amino acids forming the K196–D35 salt-bridge and some other catalytically important residues, W595, R643, and H593 and are highlighted and shown in wireframe representation. The inhibitor, Z-Pro-prolinal forming a hemiacetal to S554 is shown in black. The figures were made by Swiss PDB Viewer (4.04) and rendered with POV-Ray 3.6.