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Crystallization and preliminary X-ray analysis of porcine muscle prolyl oligopeptidase

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Abstract

Prolyl oligopeptidase from pig muscle has been crystallized in complex with an inhibitor, using PEG 8000 and calcium acetate as precipitants. The crystals are orthorombic and the space group is $P2_12_12_1$ with cell dimensions a = 111.8, b = 101.8, c = 72.4 Å. The asymmetric unit contains a single chain of prolyl oligopeptidase, corresponding to a specific volume of 2.55 Å^3 Da^{-1} and a solvent content of 52%. The observed diffraction pattern extends to 2.3 Å resolution and the native crystals are well suited for structural analysis by X-ray diffraction methods.

1. Introduction

Prolyl oligopeptidase (E.C. 3.4.21.26), previously called prolyl endopeptidase or post-proline cleaving enzyme, is a high-molecular-weight (molecular mass 80 kDa) intracellular serine peptidase (Polgár, 1994). It may be involved in the maturation and degradation of peptide hormones and neuropeptides (Wilk, 1983). Hence, it has been suggested that it plays a role in the process of learning and amnesia through its possible action on vasopressin and substance P (Yoshimoto *et al.*, 1987). Participation in the control of blood pressure is also possible through metabolism of bradykinin as well as angiotensin I and II (Welches *et al.*, 1993).

A number of pharmaceutical companies have research programmes directed toward the discovery of inhibitors of the enzyme (Tanaka *et al.*, 1994) which can be developed into potent anti-dementia drugs. Prolyl oligopeptidase is the prototype of a new serine peptidase family unrelated to trypsin and subtilisin. Although the *in vivo* function and the substrate(s) of prolyl oligopeptidase are not known with certainty, experiments with drug candidates being carried out in several laboratories will eventually address these issues.

The members of the trypsin family form a twinned β-barrel structure, while those of the subtilisin family have a tertiary structure characterized by a central β-sheet which is flanked by α-helices on both sides. Moreover, in members of the trypsin family, the relative sequence order of the catalytic triad is His–Asp–Ser, whereas, in the subtilisin family, this order changes to Asp–His–Ser. The enzymes of the prolyl oligopeptidase family represent a third relative sequence of this triad (Ser–Asp–His). These results support the view that this third serine protease class also has a different tertiary structure. Members of the prolyl oligopeptidase subfamily show no sequence homology with either the trypsin or the subtilisin family of serine proteases and it has been suggested that they constitute a new serine protease class (Rawlings *et al.*, 1991; Polgár & Szabó, 1992; Barrett & Rawlings, 1992). Recent crystallographic structure determinations of lipases have also revealed a Ser–Asp–His catalytic triad (Brady *et al.*, 1990; Winkler *et al.*, 1990) with a relative sequence order identical to that found in prolyl oligopeptidase. This has prompted speculations about the possible evolutionary and structural relationships between lipases and peptidases of the prolyl oligopeptidase family (Polgár, 1992). A secondary structure prediction (Goossens *et al.*, 1995) has also suggested that prolyl oligopeptidase has a domain with the α/β hydrolase fold, but sequence homology with proteins of this group is so low (less than 10%) that molecular-replacement efforts would not be expected to succeed.

2. Results and discussion

With the expectation of new structural features, as well as the planned utilization of the structure in drug design, we initiated the three-dimensional structure determination of prolyl oligopeptidase. For this purpose we purified the pig-muscle enzyme (Polgár, 1994). This protein could be prepared in amounts sufficient to conduct crystallization trials. Since its sequence is known to be closely related to that of the human-brain enzyme (around 97% sequence identity), the structure of the pig-muscle enzyme should be a good starting point for drug design efforts.

We crystallized the protein by vapour diffusion at 277 K using the hanging-drop method. Crystallization droplets of 6 µl initial volume were prepared on siliconized glass cover slips suspended over 1.0 ml reservoirs containing 50 m*M* MES (morpholino-ethanesulfonic acid) buffer pH 6.0, 18% PEG (polyethylene glycol) 8000 and 20 m*M* calcium acetate precipitants. The concentration of prolyl oligopeptidase was between 10 and 15 mg ml^{-1}. In most cases the bunches of crystals, which appeared in the drops after 2–3 d, looked similar to pine needles. None of the single needle-shaped crystals was larger than 0.05 × 0.1 × 0.5 mm.

Crystals were mounted in thin-walled glass capillary tubes and photographed with an oscillation camera at room temperature with graphite-monochromated Cu $K\alpha$ radiation. Unit-cell parameters were determined from five stills taken at increments of 45°. A full oscillation data set was then collected. All data were collected on a Rigaku RAXIS-II imaging-plate area detector used in connection with a Rigaku RU-200 rotating-anode generator.

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On the basis of systematic absences, Laue symmetry and the unit-cell parameters, we concluded that the crystals belong to the space group $P2_12_12_1$ with a = 111.8, b = 101.8, c = 72.4 Å. One monomer is located in the asymmetric unit resulting in a specific volume of 2.55 $Å^3\ Da^{-1}$, which is near the median of the normally observed range for globular proteins (Matthews, 1968) and corresponds to 52% solvent content.

A 2.8 Å resolution native data set was collected with $I/\sigma(I)$ of 2.7 for the highest resolution shell. Thus far, heavy-atom derivatization has always decreased the resolution considerably. In a number of cases, this has resulted in crystals that diffract to less than 10 Å resolution and has led to considerable shortening of the lifetime of the crystals in the X-ray beam. The search for heavy-atom derivatives continues.

We have also set up crystallization trials with a 1 m*M* inhibitor (*Z*-prolyl-prolinal or *N*-benzyloxycarbonyl-prolyl-prolinal) which has a K_i value in the n*M* range. Although this compound has been demonstrated to be a covalent inhibitor, bound through its C-terminal aldehyde group to the serine hydroxyl in a hemiacetal form (Kahyaoglu *et al.*, 1997), it is nevertheless also a reversible inhibitor and competitive with the substrate, since the hemiacetal bond is readily hydrolysed. In one case, trials with the inhibited enzyme provided a dramatic increase in resolution, although the crystals remained isomorphous to the native ones. At present we have a 2.3 Å data set with 95% completeness. The $I/\sigma(I)$ ratio of the highest resolution shell is 3.1 in this data set.

Determination of the three-dimensional structure of the inhibitor complex will provide a template for the rational design of prolyl oligopeptidase inhibitors.

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