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The three-dimensional structure of the bifunctional proteinase K/α-amylase inhibitor from wheat (PKI3) at 2.5 Å resolution

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Wheat germ contains an inhibitor for proteinase K, called PKI3 ($M_r \sim 19\,600$) which simultaneously inhibits α-amylase. PKI3 was crystallized, space group $P2_1$, $a = 43.02$ (5) Å, $b = 65.18$ (7) Å, $c = 32.33$ (4) Å, $\beta = 112.79°$ (9). X-ray data were collected to 2.5 Å resolution, the structure solved by molecular replacement on the basis of the atomic coordinates of the homologous *Erythrina caffra* DE-3 inhibitor, and refined with simulated annealing techniques with a current R-factor of 21%. The three-dimensional structure of PKI3 is stabilised by two disulfide bridges and has a central β-barrel with distorted β-structure. In analogy to related inhibitors, the binding site for proteinase K is assumed to be located on the surface of the protein (amino acid residues 66–67), although the 75–76 peptide bond is cleaved upon binding.

Inhibitor; Proteinase K; α-Amylase; X-ray structure; Molecular replacement; Simulated annealing

1. INTRODUCTION

Specific inhibitors are found in all kinds of tissues together with proteinases. Their function is to regulate the activity of the proteinases, thereby protecting the cells against uncontrolled proteolysis. The naturally occurring protein inhibitors for proteinases are always specific for enzymes belonging to one mechanistic class, but some of them have been reported to have additional inhibiting activity against α-amylase [1,2].

Inhibitors for serine proteases are under intensive investigation: hundreds of them were isolated, characterized and sequenced. The reaction of these inhibitors with a protease can be described by a 'standard mechanism' suggested by Laskowski [3] for soybean trypsin inhibitor (SBTI), which includes the formation of a 1:1 complex of both proteins followed by the cleavage of one peptide bond of the inhibitor. The complexes enzyme/inhibitor and enzyme/modified inhibitor are of similar stability. The cleavage of the peptide bond can be used for chemical mutagenesis of the active site of the inhibitor [4].

Up to now, the crystal structures of two serine protease inhibitors from leguminosae belonging to the Kunitz-type soybean trypsin inhibitor family have been determined: SBTI [5] and DE-3 from Erythrine caffra (DE-3) [6]. In spite of the limited sequence similarity (39% identical amino acid residues), the three-dimensional structures are very similar. Both proteins are globular with a diameter of ~35 Å. Their secondary structure is mainly of the β-type with only small portions of regular β-sheets. Six of the β-strands form a distorted β-barrel with a core of hydrophobic amino acid side chains.

We now have determined the crystal structure of a related proteinase K (PRTK) inhibitor from wheat germ, PKI3. It has 26% sequence identity with DE-3 (31% with SBTI). PKI3 consists of a single polypeptide chain with 180 amino acid residues and has a molecular mass of 19 641 Da [1]. It is also a potent inhibitor for α-amylases from wheat and from several insects. It is assumed that PKI3 has two different binding sites because the activity against α-amylase is retained after incubation with PRTK [2].

2. MATERIALS AND METHODS

PKI3 was isolated as described [7]. Crystals with dimensions 0.8 × 0.5 × 0.2 mm^3 were obtained through microdialysis against polyethylene glycol, pH 6.7, within a few days [8]. They belong to the monoclinic space group $P2_1$ with unit cell dimensions $a = 43.02$ (5) Å, $b = 65.18$ (7) Å, $c = 32.33$ (4) Å, $\beta = 112.79°$ (9).

Crystals were mounted in thin-walled glass capillaries for X-ray experiments. Intensity data up to 2.5 Å resolution were measured on a STOE four-circle diffractometer using Ni-filtered CuK_α-radiation in the $2\theta/\omega$ step-scan mode ($\lambda = 1.5418$ Å). Three reflections measured every 60 min were used to correct the data for radiation damage. The intensity data were corrected for Lorentz- and polarisation effects and a semi-empirical absorption correction [9] was applied.

The crystal structure of PKI3 was determined by molecular replacement using a model structure based on the related inhibitor DE-3 from *Erythrina caffra* whose preliminary atomic coordinates were kindly provided by S. Onesti, P. Brick and D. Blow (Imperial College of Science and Technology, London). The model structure

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was constructed by alignment of the amino acid sequences [10] and exchange of differing side chains; deletions were made, but no insertions [11]. The final search model comprised 169 out of 180 amino acid residues.

The program package MERLOT [12] was used to solve the rotation function and the translation problem. The rotation function gave a single solution. The packing function indicated only one possible molecular position which could be confirmed with the translation function. After four cycles of rigid body refinement [13] the R-value converged at was 0.53 (R_w = 0.601) for reflections between 9 and 5.5 Å resolution. The electron density was calculated and displayed on a PS300 vector graphics display [4]. The protein structure was fitted into the electron density and then divided into 13 structural domains. Rigid body refinement of these domains was carried out, the model adjusted manually and refined by simulated annealing using the program XPLOR [15]. For the first refinement cycle the resolution range was chosen between 8 and 3.5 Å with the temperature control factor set to 2000 K. The R-value after refinement was $R = 29.3\%$. At this stage several manual adjustments including omit maps were necessary to fit the polypeptide chain into the electron density. The resulting model which does not include any water molecules was again refined with XPLOR against all intensity data between 5 and 2.5 Å resolution. Individual temperature factors were assigned for all non-hydrogen atoms, with mean $B = 13.2$ Å^2. The current R-value is $R = 21\%$ for 4680 data with $|F_o| \geq 3\sigma|F_o|$[a].

3. RESULTS AND DISCUSSION

A schematic representation of the three-dimensional structure of PKI3 is shown in Fig. 1. The main structural feature is a distorted 6-stranded β-barrel consisting of antiparallel β-strands 1, 4, 5, 8, 9 and 12. The core of the β-barrel is mainly hydrophobic. Similar arrangements have also been found for e.g. papain [16] and interleukin-1β [17].

The folding of PKI3 is shown in Fig. 2. The three-dimensional structure is stabilized covalently by two disulfide bridges (cysteines 42–89 and 143–147), both on the surface of the protein and solvent-accessible. The N-terminus of PKI3 is located between two loops connecting β-strands 4 and 5, and 8 and 9, respectively, and closes the 'entrance' to the β-barrel. The C-terminus is an elongation of β-strand 12, protruding from the protein like a finger.

In spite of the low sequence homology the overall structures of PKI3 and DE3 are very similar. The largest deviations in the backbone folding are due to insertions or deletions occurring in loops about residues 72–79, 98–112, and 143–147.

PKI3 is rich in prolines which constitute 8.3% of the amino acid residues. Three of the prolines are located at the N-terminus (amino acid residues 2–4), and two near the C-terminus (amino acid residues 178–179). Prolines 63 and 68 flank the loop which contains the binding site in SBTI. The remaining prolines are located on the surface of the protein in loops connecting β-strands.

Based on this structure information, the binding site

[a]C_α-atom coordinates will be deposited in the protein data bank

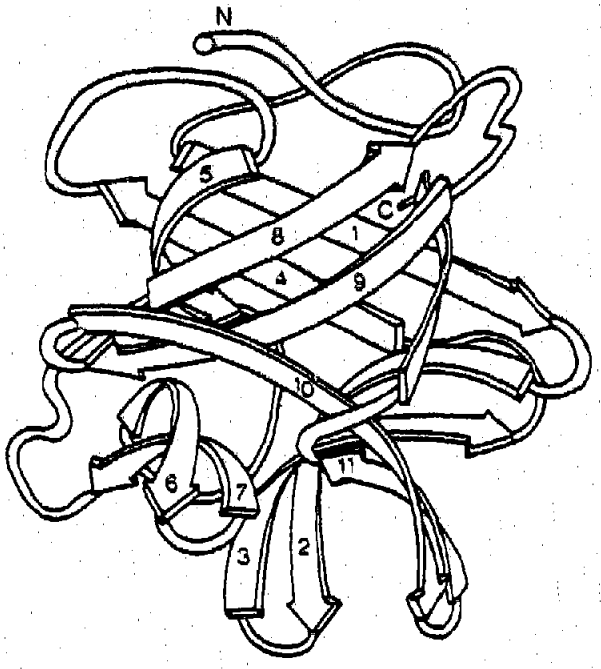

Fig. 1. Cartoon presentation of PKI3. The β-strands are related by a three-fold pseudo-symmetry. Strands 1, 4, 5, 8, 9 and 12 form an antiparallel β-barrel.

for α-amylase cannot be derived. This is because in the α-amylase inhibitors Haim II and Hoe-467A a functionally essential tryptophane is located in the binding sites with a characteristic Trp-Arg-Tyr-stack [18]. PKI3 contains only two tryptophanes which are internal and not exposed on the surface, and consequently cannot be considered as part of the functionally active site.

Incubation of PKI3 with PRTK leads to the cleavage of the Ile-75–Ser-76 peptide bond in PKI3. We have tried to model the structure of the complex between proteinase K and PKI3 with this bond in the active site. This is not possible without major changes in the conformations of either protease or inhibitor. The peptide bond in SBTI which is hydrolyzed by trypsin in the inhibition process is Arg-64–Ile-65 [19]. It is structurally equivalent to Gly-66–Ala-67 in PKI3 which is localized in a loop connecting β-strands 4 and 5 at the surface of the protein and therefore easily accessible by the protease.

The complex between proteinase K and PKI3 was crystallized and X-ray data were collected to 2.5 Å resolution. Molecular replacement techniques with the atomic coordinates of proteinase K [20] and PKI3 from this study were used to place the two molecules in the crystal symmetric unit. This led to a complex where the segment Gly-66–Ala-67 of PKI3 is located in the active site of proteinase K. This structure is under refinement and will give information concerning specific protease-inhibitor interaction and on the functional mechanism of this class of inhibitors.

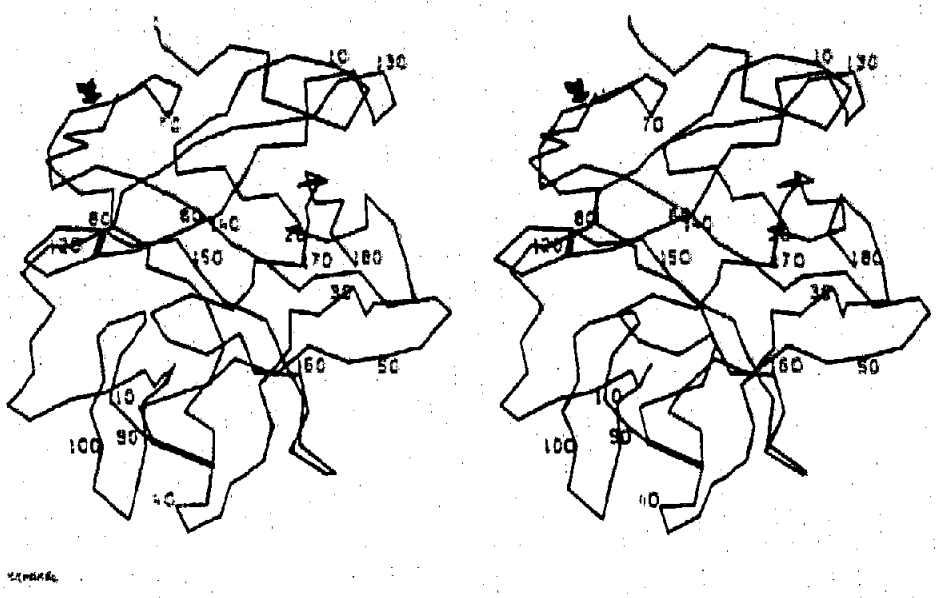

Fig. 2. Stereo plot of the Cα-backbone of PK13. The binding site for proteinase K is assumed to be located in a loop. It is marked by an arrow. Disulfide bridges are indicated by thick lines.

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REFERENCES

[1] Jany, K.-D., Ulmer, W., Fröschle, M. and Pfleiderer, G. (1984) FEBS Lett. 165, 6–10.
[2] Mundy, J., Hejgaard, J. and Svendsen, I. (1984) FEBS Lett. 167, 210–214.
[3] Laskowski Jr, M. and Kato, I. (1980) Annu. Rev. Biochem. 49, 593–626.
[4] Birk, Y. (1987) in: Hydrolytic Enzymes (Neuberger, A. and Brocklehurst, K. eds) pp. 257–305, Elsevier, Amsterdam.
[5] Sweet, R.M., Wright, H.T., Janin, J., Chothia, C.H. and Blow, D. (1974) Biochemistry 13, 4212–4228.
[6] Onesti, S., Brick, P. and Blow, D. (1990) Acta Crystalogr. A46, PS-03.11.03.
[7] Jany, K.-D. and Lederer, G. (1985) Biol. Chem. Hoppe-Seyler 366, 807–808.
[8] Pal, G.P., Betzel, C., Jany, K.-D. and Saenger, W. (1986) FEBS Lett. 197, 111–114.
[9] North, A.C.T., Phillips, D.C. and Matthews, F.S. (1968) Acta Crystalogr. A24, 351–359.
[10] Devereux, J., Haeberli, P. and Smithies, O. (1984) Nucleic Acids Res. 12, 387–395.
[11] Dayringer, H.E., Tramontano, A., Spray, S.R. and Fletterick, R.J. (1986) J. Mol. Graphics 4, 82–87.
[12] Fitzgerald, P.M.D. (1987) J. Appl. Cryst. 21, 273–278.
[13] Sussman, J.L. (1985) Methods Enzymol. 115, 271–303.
[14] Jones, T.A. and Liljas, L. (1984) Acta Crystalogr. A40, 50–57.
[15] Brünger, A.T., Kuriyan, J. and Karplus, M. (1987) Science 235, 458–460.
[16] Drenth, J., Jansonius, J.N., Koekoek, R. and Wolters, B.G. (1971) Adv. Prot. Chem. 25, 79–115.
[17] Priestle, J.P., Schär, H.-P. and Grütter, M.G. (1988) EMBO J. 7, 339–343.
[18] Pflugrath, J.W., Wiegand, G. and Huber, R. (1986) J. Mol. Biol. 189, 383–386.
[19] Sealock, R.W. and Laskowski Jr, M. (1969) Biochemistry 8, 3703–3710.
[20] Betzel, Ch., Pal, G.P. and Saenger, W. (1988) Acta Crystalogr. B44, 163–172.