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The three-dimensional structure of the complex of proteinase K with its naturally occurring protein inhibitor, PKI3

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Abstract

Proteinase K forms a 1:1 stable complex with its naturally occurring protein inhibitor, PKI3. The crystal structure of this complex has been determined by a combination of molecular replacement and single isomorphous replacement methods. The model comprises all of the 459 residues: 279 for proteinase K and 180 for PKI3, and it was refined to an *R*-factor of 19.2% at a resolution of 2.5 Å. Association of these two molecules in the complex indicates the binding of PKI3 in the substrate recognition site of the enzyme. The active serine residue of proteinase K in this complex possesses a somewhat different configuration to that found in its native structure and hence renders the enzyme inactive.

Key words: Proteinase K; Enzyme-inhibitor complex; X-ray structure; Molecular replacement; Single isomorphous replacement; Phase combination

1. Introduction

Proteinase K is a serine protease isolated from the fungus *Tritirachium album* Limber [1]. It consists of 279 amino acid residues with a molecular weight of 28,790 Da [2]. It can readily hydrolyze native proteins and remains active in the presence of urea and sodium dodecyl sulfate [3,4]. These properties make proteinase K a useful tool for the preparation of protein-free samples of DNA or RNA [5–7]. According to its amino acid sequence [2] and three-dimensional structure [8,9], proteinase K, which is of eukaryotic origin, is similar to bacterial subtilisins. It displays no significant specificity towards protein substrates but its cleavage pattern on oxidized insulin B-chain suggests that the smallest peptide hydrolyzable by proteinase K should be a tetrapeptide [10]. This was further supported by recent results of X-ray analyses of proteinase K complexed with synthetic substrate analogues, which showed that the incoming substrate forms an antiparallel β-pleated sheet structure in the substrate recognition site of the enzyme [11,12].

PKI3 is a natural inhibitor of proteinase K with which it forms a 1:1 complex with a dissociation constant of 10^{-9} M at pH 7.8 [13]. PKI3 is isolated from wheat and consists of 180 amino acids with a molecular weight of 19,641 Da [14]. It is a bifunctional inhibitor: it can inhibit serine proteases of the subtilisin family as well as α-amylases from wheat and several other cereals [13,15]. Furthermore, it was shown that the inhibitory activity of this inhibitor against α-amylase can be retained even in the presence of subtilisin, which leads to the conclusion that this inhibitor may be 'double- headed' containing separate sites directed against α-amylases and subtilisins [15].

In view of the tight association between protease and inhibitor and of the importance, both theoretical and practical, of controlling the activity of proteases, we undertook a crystallographic study of the complex between proteinase K and its inhibitor PKI3. In this paper we present the structural results that explain the association of the two molecules and the molecular details of the inhibition.

2. Experimental

2.1. Purification, crystallization and crystal properties

Proteinase K was purchased from E. Merck, Darmstadt, and was purified further according to [16]. PKI3 was isolated from wheat as described earlier [13]. A complex between proteinase K and PKI3 was prepared by mixing them in equimolar amounts. The mixture was purified by Sephadex G-75 gel filtration to remove any unreacted enzyme or inhibitor. Crystals of the complex were grown at room temperature from 25% ammonium sulfate in 50 mM Tris-HCl, pH 7.8. Large single crystals of an average size of $0.2 \times 0.4 \times 1.5$ mm^3 were obtained by vapor diffusion. They diffracted X-rays to 2.5 Å resolution. The crystals belong to the orthorhombic space group $P2_12_12_1$ with cell dimensions $a = 64.07$ Å, $b = 66.84$ Å and $c = 133.82$ Å.

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2.2. Data collection and processing

Diffraction data of the native and one heavy atom derivative crystals were collected in house with Siemens/Nicolet X-100A area detector on an Elliott GX18 rotating anode with monochromatized CuKα radiation. Crystals were mounted as c^* along the spindle axis and oscillated for 0.25° per frame. Data processing was performed using the program XDS [17]. The results are summarized in Table 1. For heavy atom derivative preparation, a single crystal was soaked at room temperature for 2 days in 1 mM solution of $HgCl_2$ in 50 mM Tris-HCl at pH 7.8 containing 25% saturated ammonium sulfate.

2.3. Structure determination

The structure of the proteinase K– PKI3 complex was solved by a combination of molecular replacement (MR) and single isomorphous replacement (SIR) methods. In order to solve the structure of the enzyme part, refined coordinates of proteinase K at 1.5 Å resolution were used as a search model [9,18] and the MR was carried out using the program package MERLOT [19]. In both the rotation and translation searches, single solutions were obtained using the native X-ray data between 8.0 and 4.5 Å resolution with $I \geq 3\sigma_I$. This unique solution was then optimized by an *R*-factor minimization and was confirmed through an intermolecular contact search as implemented in the MERLOT program package.

In a similar way, an attempt was also made to determine the structure of the inhibitor part of the complex using PKI3 coordinates determined at 2.5 Å resolution [20]. However, this was unsuccessful, probably due to the fact that PKI3 constitutes only 40% of the complex. On the other hand, MR phases alone could not generate an interpretable electron density map in which the inhibitor model could be built.

Thus to obtain the structure of the inhibitor part, SIR was carried out using the Hg derivative of the complex crystals. All the calculations were performed with the CCP4 program package. In a difference Patterson map only a single heavy atom binding site was interpretable, which was confirmed by calculating a difference Fourier map using the MR phases. Table 2 summarizes the heavy atom parameters. Based on this single site, phases were calculated and the resulting SIR phases were then combined with the MR phases using the program SIGMAA as implemented in the CCP4 program package. An overall figure of merit of 0.66 was obtained for reflections between 5.0 and 2.71 Å resolution.

Electron density maps were calculated using the combined phases as well as MR phases with the coefficients $2F_o$-F_c and F_o-F_c, and based on these maps the inhibitor chain was traced on an Evans and Sutherland picture system using the program FRODO [21].

2.4. Model building and refinement

An electron density map generated with the combined phases led to some minor corrections for the enzyme part; however, the inhibitor model had to be built in a series of steps. At first it was possible to fit as many as 131 out of 180 amino acid residues of the inhibitor, PKI3, and the initial *R*-factor of 37.4% with the enzyme only dropped to 31.3% at this stage. In each of the subsequent steps, after manual fitting the model was refined for a few cycles with the program PROLSQ [22] considering the data between 5.0 and 3.0 Å resolution. However, maps were generated with all the reflections ($I \geq 3\sigma_I$) using the model biased phases. In the final step the model consisting of the complete enzyme and 164 residues of the inhibitor gave an *R*-factor of 22.8% with the data where $I \geq 3\sigma_I$. It was then submitted for further refinement with the program XPLOR [23] adding more reflections up to 2.5 Å resolution and $I \geq 2\sigma_I$. After two cycles of positional refinement followed by one cycle of simulated annealing, it was possible to fit all the residues in the electron density map and the refinement was continued thereafter followed by individual temperature factor refinement. Fig. 1 illustrates a typical section of the final map.

The current model, which comprises all the 459 residues (279 for proteinase K and 180 for PKI3) but without solvent molecules, gives an *R*-factor of 19.2% (considering the reflections between 5.0 and 2.5 Å resolution and with $I \geq 2s_I$) after assigning individual temperature factors for all non-hydrogen atoms. Refinement of the individual *B*-factors was carried out using an XPLOR protocol [24]. *B*-factors obtained for main chain atoms lie within the range 6.3 to 30.0 Å^2 with a mean of 14.1 Å^2, and for side chain atoms between 6.5 and 31.1 Å^2 with a mean of 14.7 Å^2. However, a few non-glycine residues, mostly of the inhibitor and in the external loops only, were found outside the energetically favoured regions in a Ramachandran Plot [25]. In the present model root-mean-square deviations in bond length and in bond angle are 0.018 Å and 3.82°, respectively.

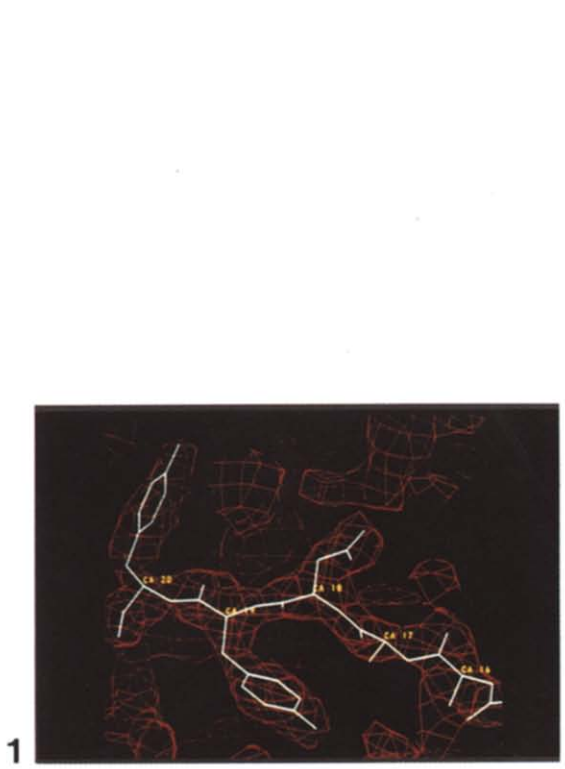
3. Results and discussions

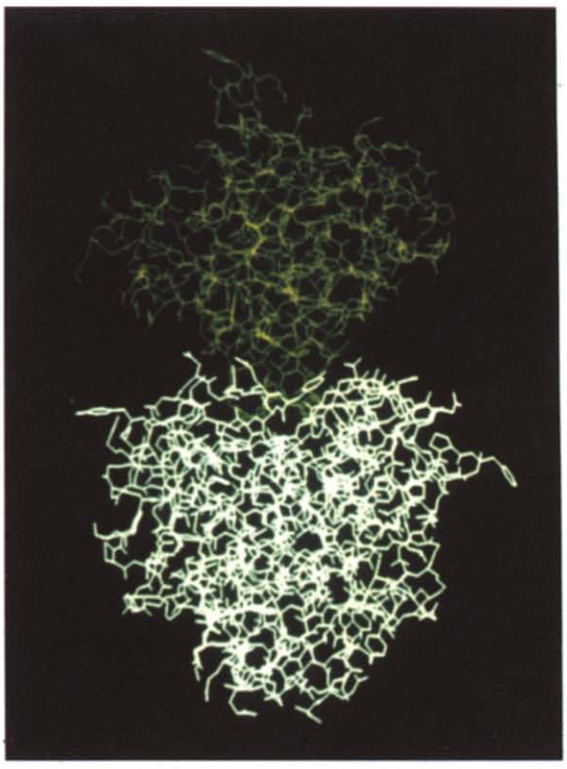
Fig. 2 shows the association of proteinase K and PKI3 in the crystal structure of their complex. Proteinase K resembles a hemisphere with a crevice on the flat surface where the active center is located. PKI3 is more like a cone in its shape and the conical tip is inserted into the crevice of the enzyme, binds there through non-covalent interaction and forms a stable complex.

Though a few corrections involving only side chains were necessary, the folding of the enzyme molecule in the complex is essentially the same as that of native proteinase K [9]. However, major conformational changes are observed in the inhibitor part if it is compared with the corresponding structure of PKI3, determined independently [20]. When these two independently determined structures of proteinase K and PKI3 were superimposed with the corresponding structures in the complex using the common α-carbon atoms, the root-mean-square deviations in the aligned positions were 0.3 Å and 3.75 Å for proteinase K and PKI3, respectively. Furthermore, the mean value of the individual temperature factors of all non-hydrogen atoms of this complex is calculated as 14.4 Å^2. The value for proteinase K alone is 11.9 Å^2 whereas for PKI3 alone it is 18.2 Å^2. These results suggest that the inhibitor is more flexible; alternatively it may be claimed that the difference in the values is due to the more accurate determination of the enzyme part of the complex. Similar results were also obtained for subtilisin–eglin crystal structures [26,27].

A closer view of the assembly of these two molecules in the complex, involving the active center of the enzyme and its binding with the inhibitor, is shown in Fig. 3. The inhibitor peptide involving residues Ala-85 to Thr-88 (the numbers 1–180 are used for the 180 residues of PKI3 and 181–459 for proteinase K) forms a three- stranded antiparallel β-pleated sheet with the two parallel strands, Gly-280 to Tyr-284 and Leu-313 to Gly-316, of proteinase K and they are held together by a strong hydrogen-bonding network. For a proper orientation of this part of the inhibitor a significant rearrangement occurred in the substrate recognition pocket of the enzyme. In particular the position of the Tyr-284 side chain now stacks together with the side chain of Tyr-86 (of PKI3) with a rotation of about 90° to each other, contributing to their tight binding in the complex. Distance calculations show that the inhibitor in this active center region is further stabilized through many other intermolecular as well as intramolecular hydrogen bonding, involving both the main chain and side chain atoms.

The catalytic triad is grossly the same but NE2 of His-249 is 3.92 Å from OG of Ser-404. This would mean





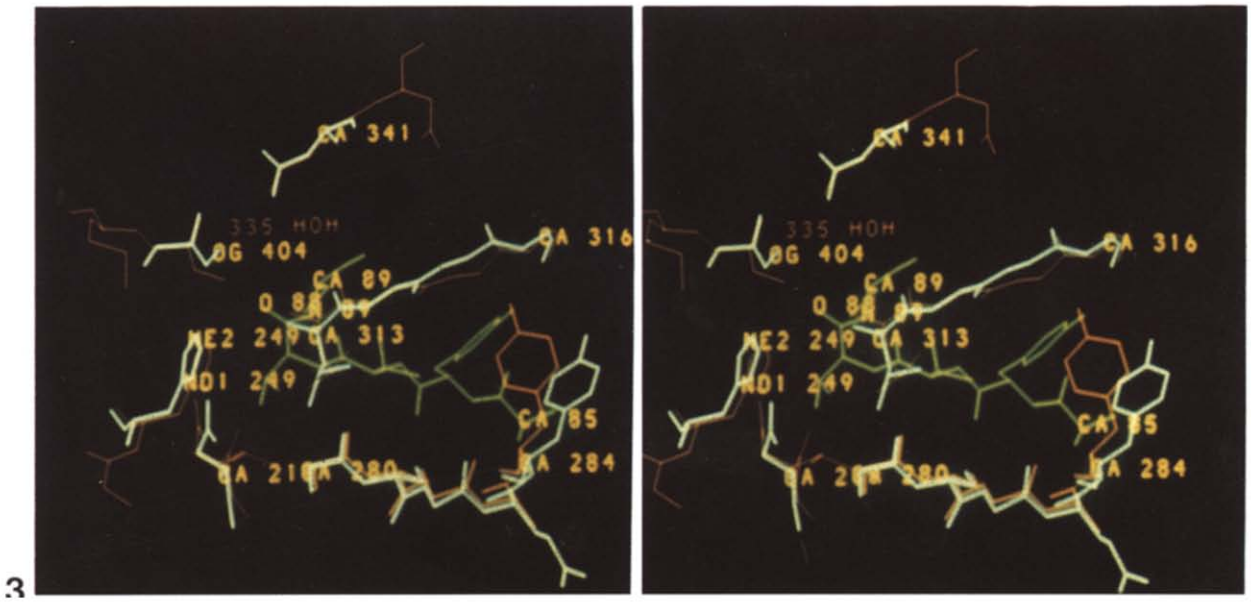


Fig. 1. A section of the final $2F_o$-F_c electron density map showing the fit of the residues Asp-16 to Tyr-20 of the inhibitor PKI3.
Fig. 2. Association of PKI3 (green) and proteinase K (white) in the crystal structure of their complex.
Fig. 3. A stereo-view of the binding of PKI3 (green) to proteinase K. The catalytic center and substrate recognition pocket of the enzyme as found in the complex (white) are superpositioned on the relevant part of the native enzyme structure (brown). Water 335 of the native structure is labelled.

that the histidine cannot take the proton of the serine, a step necessary for the proteolytic activity of the enzyme through nucleophilic attack by the deprotonated serine. The NE2 of His-249 is now hydrogen-bonded with the Thr-88 carbonyl oxygen of the inhibitor and stabilizes it further. The OG of Ser-404 turns almost 90° to a position where it is now hydrogen bonded with the side chain of Asn-341 and this position is equivalent to the 'oxyanion hole' in the native structure of the enzyme where a water molecule was located [9]. In the native structure, this water molecule, which was considered to be involved in the deacylation process of the enzyme–

Table 1
Data collection summary for native and heavy atom derivative crystals

	Native	$HgCl_2$
Resolution (Å)	2.5	2.8[a]
Total observations	57,576	39,996
Unique reflections	19,462	14,516
Completeness (%)	93	89
R_{merge} (%)[b]	5.92	5.6

[a]16.7% of the possible data are also observed in the 2.5–2.8 Å resolution.
[b]$R_{merge} = (\sum_h |(< I_h > - I_h)|/\sum_h I_h) \times 100$
where $< I_h >$ is the mean of the I_h observations of reflection h.

substrate complex, was hydrogen bonded with the side chain of Asn-341. Although the present structure has not yet been fully analyzed with respect to solvent, no electron density was observed in this position in the difference map, which would correspond to a water molecule. The change in position of Ser-404 was not observed in the structures of the enzyme with substrate analogues [11,12] and with hexapeptide inhibitor [28].

In a previous paper [20], cleavage of PKI3 by proteinase K has been reported. However, that occurs at or near pH 6.0. Under the experimental conditions of our biochemical investigation, i.e. at pH 7.8 we did not observe any such cleavage. Moreover, in this present crystallographic study we can see the whole molecule of PKI3 in the assembly of the complex and there is no sign of cleavage. The lack of cleavage is further supported by the fact that the distance from the oxygen of the active serine to the carbon of the putative scissile bond between Thr-88 and Cys-89 is 4.62 Å. However, the corresponding distance in the complex of proteinase K with its hexapeptide inhibitor where cleavage does occur is 2.11 Å [28].

In summary our result shows that, upon binding to the enzyme, the inhibitor is placed on the active site and pushes the oxygen of the active serine to form a hydrogen bond with Asn-341 where it can not be the part of the active catalytic triad.

Addition of solvent and further refinement are now in progress.

Table 2
Refined heavy-atom-parameters of the $HgCl_2$ derivative

Fractional coordinates,	x	0.1061
	y	0.0108
	z	0.0648
Relative occupancy		3.15
Temperature factor		31.8
Cullis R-factor		0.511

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