Inhibition of Proteinase K by Methoxysuccinyl-Ala-Ala-Pro-Ala-chloromethyl Ketone

AN X-RAY STUDY AT 2.2-Å RESOLUTION*

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The crystal structure of the transition state analog complex formed covalently between proteinase K and methoxysuccinyl-Ala-Ala-Pro-Ala-chloromethyl ketone was determined by x-ray diffraction methods at a resolution of 2.2 Å and refined by constrained least squares to an R factor of 19.8% for the 11864 structure amplitudes greater than 1σF. The chloromethyl ketone group is covalently linked with the active site functional groups His67(N.) and Ser224(O.). The former has substituted for chlorine and the latter has attacked the carbon of the ketone group, thereby forming the tetrahedral carbon atom of the transition state analog. The peptide part of the inhibitor is in an extended conformation and fills subsites S1 to S5 of the substrate recognition site. Its backbone hydrogens bond with histidine and serine residues in the ubiquitous serine proteases in a way similar to the already known shorter inhibitors (4, 5), fits completely into the substrate binding site even if the amino acid in the subsite S2 (according to the Schechter and Berger nomenclature (8)) is a proline, followed by two to four amino acids with small side chains. To test this suggestion obtained from model building studies, the inhibitory power against proteinase K of the commercially available N-terminal protected methoxysuccinyl-Ala-Ala-Pro-Ala-chloromethyl ketone was investigated by kinetic assays. Since it strongly inhibits proteinase K, the two molecules were cocystalized and the structure of the complex determined by x-ray diffraction methods.

MATERIALS AND METHODS

Proteinase K was obtained from SERVA, Heidelberg, and purified by Sephadex G-50 gel filtration in 50 mM Tris-HCl pH 7.5, containing 1 mM CaCl₂ to remove autolytic fragmetns, as previously described (9), and lyophilized. The inhibitors Ala-Ala-COCH₂Cl (I), Ala-Ala-Phe-COCH₂Cl (II), Phe-Pro-Arg-COCH₂Cl (III), methoxysuccinyl-Ala-Ala-Pro-Ala-COCH₂Cl (IV) were purchased from SERVA, Heidelberg, and from Calbiochem, Bubendorf (Switzerland), and used without further purification. The latter inhibitor is soluble in water, and the others were first dissolved in a minimum volume of methanol and then added to aqueous buffered solutions of proteinase K. For inhibition studies, inhibitors I-III were dissolved 0.5 µg/µl in 50 mM Tris-HCl, 1 mM CaCl₂, pH 8.0, containing 60% methanol. Stock solutions of proteinase K (0.2 µg/µl) and of the substrate succinyl-(Ala)-CO-NH-(C₆H₄)-NO₂ (1 mM) were prepared in the same buffer without methanol; in all experiments the molar ratio of inhibitor to enzyme was 100:1. Assays contained 0.7 nmol of proteinase K, 70 nmol of inhibitor, 0.75 µmol of substrate, 0.04-4% methanol. After enzyme and inhibitor were incubated for 15 min at 25°C, the substrate succinyl-(Ala)-CO-NH-(C₆H₄)-NO₂ was added and reacted 1 h. The reaction was then stopped with glacial acetic acid and the released p-nitrophenyl ketones were defrayed in part by the payment of page charges. This article was therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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The inhibition of proteases by specifically designed molecules is of particular interest in view of the involvement of these enzymes in biological processes which frequently are of medical importance. One class of inhibitors, the peptide chloromethyl ketones, has been more intensively studied for two reasons: (i) they bind covalently to the catalytically active site residues His67 and Ser224 in a way similar to the already known shorter inhibitors (4, 5), fits completely into the substrate binding site even if the amino acid in the subsite S2 is a proline, followed by two to four amino acids with small side chains. To test this suggestion obtained from model building studies, the inhibitory power against proteinase K of the commercially available N-terminal protected methoxysuccinyl-Ala-Ala-Pro-Ala-chloromethyl ketone was investigated by kinetic assays. Since it strongly inhibits proteinase K, the two molecules were cocystalized and the structure of the complex determined by x-ray diffraction methods.
TABLE I

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Relative inhibition rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Ala-Ala-COCH₂Cl</td>
<td>10</td>
</tr>
<tr>
<td>II Ala-Ala-Phe-COCH₂Cl</td>
<td>28</td>
</tr>
<tr>
<td>III Phe-Pro-Arg-COCH₂Cl</td>
<td>10</td>
</tr>
<tr>
<td>IV Methoxysuccinyl-Ala-Pro-Ala-COCH₂Cl</td>
<td>88</td>
</tr>
</tbody>
</table>

TABLE II

**Data collection, data processing, and refinement statistics**

Crystal habit tetragonal bipyramids; space group P4₁2₁2₁; cell constants a and b = 68.3(2) Å, c = 108.4(2) Å, CuKα radiation (λ = 1.5418 Å) from Elliott GX20 rotating anode generator, 40 kV, 70 mA, 0.2 × 2 mm² focus; Cea-Reflect 25 (Ceaverkaen) films in 3-packs; 59-mm film-to-crystal distance, 2.2 Å resolution; crystal mounted along c; 1.5° oscillation per exposure of 50 min; 53620 reflections collected, merged to 11864; overall merging Rmerge = 9.8%; mean B factor from Wilson plot = 14.6 Å².

Refinement statistics; rms deviations from ideal values

| Bond lengths (Å) | 0.009 |
| Bond angles (°)  | 1.824 |
| Torsion angles (°) | 24.470 |
| Trigonal atoms planarity (Å) (carboxylates, amides, peptides) | 0.017 |
| Planar groups (Å) | 0.025 |
| Bad contacts (Å)  | 0.074 |
| Chirality restrained to L-configuration for all amino acids | 1.824 |

Final R factor² for 11.864 reflections | 0.198 |

Mean temperature factor B = 15.0 Å²

mol⁻¹ grew within 1 day at room temperature.

The crystals of diameter ~0.8 mm are isomorphous to crystals of native proteinase K (10, 11). X-ray data collection to 2.2 Å resolution was carried out by film methods using an Arndt-Wonacott rotation camera installed on an Elliott GX20 rotating anode x-ray generator (see Table II). The x-ray intensities on the films were digitized with an Optronics P1000 film scanner and processed with the MOSFAC program system (12).

The coordinates of all the protein and two Ca²⁺ atoms of the 1.5-Å resolution structure of proteinase K (11) were used as a starting model in the structure determination. For the preliminary stages of restrained least-squares refinement (13), only the 7809 data >3σ were included in the 5–2.2 Å resolution shell were used, with an initial R factor of 0.283. After 8 cycles of refinement, the R factor converged at 0.198. The subsequent refinement was carried out with the reciprocal space least-squares program FRODO (6) by means of an Evans and Sutherland PS350 graphics display. The map clearly revealed the Ala-Ala-Pro-Ala portion of the inhibitor and the covalent bonds of the -CO-CH₂- group with His⁹⁰(N₃) and Ser²⁴⁰(O₅), but the methoxysuccinyl group could not be located. A further 5 cycles of refinement with restrained covalent enzyme-inhibitor bonds led to a map in which electron density for the protecting group could be interpreted (Fig. 1). The subsequent refinement was carried out with the reciprocal space least-squares program TNT (14) because it allows for better definition of the restraints within the inhibitor binding site. At this stage all the 11864 x-ray data above the 1σ level were successively included and the resolution range extended to 10 Å. Inspection of 2Fₐ-Fc maps not only allowed for improvement of the position of the inhibitor, but also to locate 170 water molecules and the terminal six atoms of the Arg⁹⁷ side chain which were not defined in the structure of native proteinase K. The strong Ca²⁺ binding site CaI is fully occupied but the site residues His⁹⁰(N₃), His⁹¹(N₆), and Arg⁹⁷(N) restrict the orientation of this group so that the C₅₅(O') oxygen cannot fully move into the "oxyanion hole" which is formed by Ser²³⁸(N), Asn²⁴⁰(N), and Asn²⁴⁰(N). In the native proteinase K, this hole is filled by a water molecule (11). Instead, the Ala²⁴⁰(O') atom forms hydrogen bonds only to Ser²³⁸(N), and because it cannot move further into the "oxyanion hole" to approach and to bind to Asn²⁴⁰(N) and Asn²⁴⁰(N), the resulting gap is filled by a well defined water molecule Wat⁶⁶ (B = 23.4 Å²) in hydrogen bonding distance, see Table III.

The covalent binding geometry parameters within the residues His⁹⁰, Ser²³⁸, and Ala²⁴⁰ were refined with constraints. As expected, the bond angles around atom Ala²⁴⁰(C) are in the range 107–112°, the angle at Ser²³⁸(O₅) is 107°, and that at the -CH₂ atom Ala²⁴⁰(C) is 116°; the CH₂-N bond distance atoms, 31 inhibitor atoms, 170 water oxygen atoms, and one Ca²⁺ atom is 0.198, based on the 11864 reflections with F > σ(F).

**RESULTS AND DISCUSSION**

In the previous crystallographic studies on native proteinase K at 1.5-Å resolution (11) and on proteinase K complexed with the inhibitor carbobenzoxy-Ala-Ala-COCH₂Cl (4), the catalytic site was identified as the triad Asp⁶⁰-His⁹⁰-Ser²³⁸, with the free Cys⁴⁶(SH) near the imidazole ring of His⁹⁰. The substrate recognition site is formed by the two peptide chains, 99–104 and 132–134, respectively (Fig. 2, a and b). These peptide chains are oriented approximately parallel and directly connected only by a hydrogen bond formed between the Tyr²⁰⁰(O₇) and Gly²⁰¹(O) (Fig. 2a). The previously studied peptide inhibitors (4, 5) and the one presently investigated are inserted into the recognition site such that an antiparallel three-stranded pleated sheet is formed. The sheet has a commonly observed overall left-handed twist.

In the reaction between proteinase K and the inhibitor methoxysuccinyl-Ala²⁸¹-Ala²⁸²-Pro²⁸³-Ala²⁸⁴-COCH₂Cl, the active site residues His⁹⁰(N₃) and Ser²³⁸(O₅) react with the terminal -CO-CH₂Cl group, to form a covalently bonded complex with proteinase K as shown in Fig. 2b. This complex mimics the transition state with tetrahedral C₅₅(O') atom, except for the -CH₂- group which, in the transition state, would be the -NH group. The -CH₂- group restricts the orientation of this group so that the C₅₅(O') oxygen cannot fully move into the "oxyanion hole" which is formed by Ser²³⁸(N), Asn²⁴⁰(N), and Asn²⁴⁰(N). In the native proteinase K, this hole is filled by a water molecule (11). Instead, the Ala²⁴⁰(O') atom forms hydrogen bonds only to Ser²³⁸(N), and because it cannot move further into the "oxyanion hole" to approach and to bind to Asn²⁴⁰(N) and Asn²⁴⁰(N), the resulting gap is filled by a well defined water molecule Wat⁶⁶ (B = 23.4 Å²) in hydrogen bonding distance, see Table III.

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![Figure 1](image)
Protein atoms as in the proteinase site substrate binding site and the catalytic lenty bound inhibitor methoxysuccinyl-Ala-Ala-Pro-Ala-C(O-)-CHz-CHz. inhibitor atoms; orange, Cys; black, water molecules; green, hydrogen bonds; blue, protein atoms as in the proteinase K-inhibitor complex; brown, as in free and fully active proteinase K. C-atom are molecules; labeled. AlaB1, and P5 for succinyl 280. Drawn in Fig. 2. a. stereo view showing the substrate binding site and the catalytic site (left) of proteinase K, and the covalently bound inhibitor methoxysuccinyl-Ala-Ala-Pro-Ala-C(O-)-CHz-. Red, inhibitor atoms; orange, Cys; black, water molecules; green, hydrogen bonds; blue, protein atoms as in the proteinase K-inhibitor complex; brown, as in free and fully active proteinase K. C-atom are molecules; labeled. AlaB1, and P5 for succinyl 280. Drawn with SCHAKAL (16).

Fig. 2. a. stereo view showing the substrate binding site and the catalytic site (left) of proteinase K, and the covalently bound inhibitor methoxysuccinyl-Ala-Ala-Pro-Ala-C(O-)-CHz-. Red, inhibitor atoms; orange, Cys; black, water molecules; green, hydrogen bonds; blue, protein atoms as in the proteinase K-inhibitor complex; brown, as in free and fully active proteinase K. C-atom are molecules; labeled. AlaB1, and P5 for succinyl 280. Drawn with SCHAKAL (16).

TABLE III
Hydrogen bonds D-H...A between inhibitor, water molecules, and proteinase K

<table>
<thead>
<tr>
<th>H atoms involved</th>
<th>D...A</th>
<th>H...A</th>
<th>D-H...A</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSU(O) - Tyr(N)</td>
<td>3.31</td>
<td>2.28</td>
<td>159</td>
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<tr>
<td>MSU(O) - Wat</td>
<td>2.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly(N) - Wat</td>
<td>3.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala11(N) - Gly(O)</td>
<td>2.72</td>
<td>2.26</td>
<td>103</td>
</tr>
<tr>
<td>Ala12(O) - Gly(N)</td>
<td>3.47</td>
<td>2.77</td>
<td>122</td>
</tr>
<tr>
<td>Ala21(N) - Gly(O)</td>
<td>3.07</td>
<td>2.11</td>
<td>147</td>
</tr>
<tr>
<td>Ala22(O) - Gly(N)</td>
<td>3.14</td>
<td>2.21</td>
<td>143</td>
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<tr>
<td>Ala23(N) - Wat</td>
<td>3.17</td>
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<td>Pro28(O) - Wat</td>
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<td>Glu29(N) - Ser24(O)</td>
<td>2.86</td>
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<td>Ala25(N) - Ser23(O)</td>
<td>2.93</td>
<td>1.86</td>
<td>168</td>
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<tr>
<td>Ala26(O) - Ser24(N)</td>
<td>2.71</td>
<td>2.00</td>
<td>142</td>
</tr>
<tr>
<td>Ala27(Wat)</td>
<td>2.84</td>
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<td></td>
</tr>
<tr>
<td>Asp151(N) - Wat</td>
<td>3.24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Additional hydrogen bonds stabilizing the active site geometry

Gly26(O) - Tyr104(N) | 2.79 |
Asp27(O2) - His151(N2) | 2.69 |
Asp27(O2) - His151(N2) | 2.91 |

MSU, methoxysuccinyl.
Wat, water.

is 1.4 Å, the C-O distances are 1.35-1.36 Å, and the C-C distances fall in the range 1.50-1.55 Å. The inhibitor adopts an extended conformation with trans Pro285, and all Φ, Ψ angles correspond to the β-pleated sheet region in a Ramachandran plot (Ala28: Φ = -100°, Ψ = 178°; Ala282: Φ = 146°, Ψ = 163°; Pro282: Φ = -80°, Ψ = 151°; Ala248: Φ = -125°). In addition to the covalent bonds with active site residues Ser224 and His289, the inhibitor is anchored in the substrate recognition site by several hydrogen bonds, details are given in Table III.

These hydrogen bonds involve, in subsite P1 (for definition see caption Fig. 2b). Ala284(N); it donates an unsymmetrical bifurcated three-center bond where the N-H opposes two oxygen atoms (15). The major component is to Ser224(O) and the minor (weaker) component to Ser224(O). In subsite P2, the oxygen Pro285(O) accepts a hydrogen bond from water Wat which simultaneously interacts with Ala284(O), and the latter oxygen accepts a second hydrogen bond from Gly134(N). Pro285 is not only held in its positions by the hydrogen bonds, but it is also fixed by van der Waals contacts with the peptide carbonyl group of Gly134 (Pro285(C) - Gly134(O), 2.98 Å). It even appears that this interaction pushes away one of the recognition strands Gly100-Tyr104 to relieve steric clash, as shown in Fig. 2a. With other amino acids in place of Pro285 this interaction is not possible because in nonproline amino acids the side chains can adopt other conformations to avoid steric clash. In addition, the N-C bond is replaced by N-H, and another hydrogen bond can be formed with the recognition site of the proteinase K. At positions P3 and S3, Ala281 and Gly284 form a hydrogen bonding motif characteristic for antiparallel β-pleated sheets, and a similar interaction is also observed for P4 and S4, formed by Ala281 and Gly102. Fig. 2, a and b. The N-terminal methoxysuccinyl group of the inhibitor is bound to the recognition site of proteinase K by two hydrogen bonds, one directed between Tyr104(N) and the ester -O-CH3 oxygen, the other mediated by a water molecule, succinylpeptide (C=O) - Wat551 - (HN) Gly139. The succinyl -NH-CO-CH2-CH2- moiety is stacked on the Tyr104(phenyl group, with intermolecular C...C distances in the range of 3.22-3.43 Å.

To detect any conformational changes that occurred upon binding of the inhibitor to proteinase K, the main chain atoms N, Cα, C, O of the complex were superimposed by least squares fitting on the corresponding atoms of the native proteinase K structure using the OVERLAY procedure of the TNT program (14). The root mean square (rms) deviations of the 1962 superimposed atoms and all the 2017 common atoms were 0.21 and 0.41 Å, respectively. Except for the binding region,
no systematic and significant conformational changes were found. Close to the active site and just "below" the plane of functionally important His imidazole, there is the free SH group of Cys (Fig. 2a), the role of which is not yet understood. Its sulfur atom is engaged in several close contacts with active site residues (His, Ser, Ser) which do not change significantly their positions after inhibitor binding except for the distance to His, which increases by 0.7 Å.

This increase in distance is caused by a movement of His which is necessary to permit formation of the covalent bond between the His(N) and the inhibitor -CH₂ group by which is necessary to permit formation of the covalent bond. When the inhibitor binds into the recognition site, strand 100–104 is consequently pushed away by ~1 Å to open the binding cleft, probably caused by the tight interaction with the side chain of the inhibitor proline, Pro(C₁)…(O)Gly, 2.98 Å.

The inhibition of proteinase K by the four different peptide chloromethyl ketone inhibitors I to IV is described in Table I. The data indicate that the inhibition (binding of the inhibitors) is better with longer peptide chains of the inhibitors. The sample of inhibitors given in Table I is not sufficient to propose a correlation between inhibitor sequence and binding affinity, but it is clear from this x-ray study that proline interferes with peptide binding to proteinase K. It suggests that more effective inhibitors would have the proline substituted by another amino acid to avoid the steric interference with strand 100–104 of the recognition site.

CONCLUSIONS

Complementary to our earlier publications on the crystal structures of complexes between proteinase K and dipeptide inhibitors carbobenzyloxy-Ala-Ala-C(O)CH₂Cl and carbobenzyloxy-Ala-Phe-C(O)CH₂Cl, we have now investigated a tetrapeptide which, in contrast to the earlier, shorter inhibitors saturates the whole substrate binding site. There is antiparallel β-pleated sheet formation with all the amino acids of the inhibitor engaged in hydrogen bonding except for Pro. If this proline were replaced by another amino acid, one more hydrogen bond Pro(C₁)…(O)Gly should be possible after minor conformational changes around position 283 of the inhibitor and/or of the protein segment Gly-Tyr.

The contact surface between the inhibitor and the substrate binding site is extensive. Besides the hydrogen bonds, it involves van der Waals interactions between the side chains of the Ala, Pro, and the substrate recognition site, and the stacking between the succinyl moiety and the Tyr phenolic group. As the "bottom" of the proteinase K substrate recognition site is predominantly hydrophobic due to the side chains of Leu and Tyr, it is not the sequence of the substrate that is of importance in the recognition but only the actual size of the side chains. Even larger side chains can be tolerated due to the flexibility of the segment Gly-Tyr, as shown with Pro of the peptide inhibitor used in this study. In this light, the observation that the affinity of proteinase K correlates with the length of the inhibitor is readily
explained as the binding to the substrate recognition site will be the stronger the more peptide-peptide hydrogen bonds are formed between inhibitor and enzyme, and the more hydrophobic contacts are made. Any sequence dependence should be of minor influence, as actually shown by the unspecific nature of proteinase K which has only some preference for aromatic, bulky amino acid side chains.

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REFERENCES