Crystal Structure of Calcium-free Proteinase K at 1.5-Å Resolution*

(Received for publication, June 6, 1994)

Alexander Müller†, Winfried Hinrichs, Wojciech M. Wolfs*, and Wolfram Saenger†

From the Institut für Kristallographie, Freie Universität Berlin, Takustrasse 6, 14195 Berlin, Federal Republic of Germany and §Daresbury Laboratory, Daresbury, Warrington WA4 4AD, United Kingdom

Proteinase K from the fungus Trichirachium album Limber binds two Ca2+ ions, one strongly (Ca1) and the other weakly (Ca2). Removal of these cations reduces the stability of proteinase K as shown by thermal denaturation, but the proteolytic activity is unchanged. The x-ray structures of native and Ca2+-free proteinase K at 1.5-Å resolution show that there are no cuts in the polypeptide backbone (i.e. no autoysis), Ca1 has been replaced by Na+, while Ca2 has been substituted by a water associated with a larger but locally confined structural change at that site. A small but concerted geometrical shift is transmitted from the Ca1 site via eight secondary structure elements to the substrate recognition site (Gly106-Tyr104 and Ser132-Gly136) but not to the catalytic triad (Asp99, His69, Ser234). This is accompanied by positional changes of localized waters.

Proteinase K (EC 3.4.21.14) from the fungus Trichirachium album Limber belongs to the subtilisin family (1-3). The x-ray structures of native proteinase K at 1.5-Å resolution (4) and in complex with several peptide chloromethyl ketone inhibitors have been determined (5, 6). The active site consists of the catalytic triad Asp99-His69-Ser234, and the substrate recognition site is formed by Gly106-Tyr104 and Ser132-Gly136 which form a triple antiparallel β-strand with the substrate. The native enzyme contains two Ca2+ sites, 17 Å (Ca1) and 26 Å (Ca2) away from the active site. They are not involved in the catalytic mechanism and contribute to the structural stability of the enzyme (3). Ca1 is tightly bound with a dissociation constant of (1.6 ± 2.5) × 10−3 M (7), and coordinated in the form of a pentagonal bipyramidal by Asp99 (Oγ1, Oγ2), the carbonyl oxygens of Pro118 and Val177, and four water molecules. Ca2 is bound with lower affinity and coordinated by Asp99 (Oγ1, Oγ2), the carbonyl oxygen of Thr86, and two water molecules (4).

If proteinase K is depleted of Ca2+, its activity was reported to drop by 80% within 6 h and could not be fully restored by readdition of Ca2+, although this is not accompanied by autoysis (7). A crystallographic study at 2.2-Å resolution suggested structural changes upon Ca2+ removal (8), with one strand of the recognition site (Gly106-Tyr104) shifted by 0.8 Å, the Ser234(Oγ1)...-His69(Nε) hydrogen bond increased by 0.5 Å, and

*This work was supported in part by the Deutsche Forschungsgemeinschaft through the Leibniz Program and Sonderforschungsbereich 9 (Teilprojekt A7) to W.S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Supported by Short-term Fellowship ASTF 6806 from the European Molecular Biology Organization.

‡Supported by a fellowship from the Alexander von Humboldt Stiftung. Present address: Inst. of General Chemistry I-17, Technical University of Lodz, ul Zwirki 36, 90904 Lodz, Poland.

To whom correspondence should be addressed.

rearrangement of water molecules. These findings were discussed as reasons for the reduced enzymatic activity. To investigate the function of Ca2+ in more detail, we determined the x-ray structure of the Ca2+-free proteinase K at 1.5-Å resolution to compare the water positions of both structures. In addition, spectroscopic studies and enzyme kinetics were carried out to characterize the influence of Ca2+ on the stability and activity of the protein. The results are reported in this article.

MATERIALS AND METHODS

Crystallography—Proteinase K was obtained from Merck, further purified, and depleted of Ca2+ as described in Ref. 7. For crystallography, the lyophilized protein was dissolved at 6% (w/v) in buffer (50 mM Tris-HCl, 300 mM sodium-EDTA, 0.02% NaN3, pH 7.5) with special care to avoid air bubbles, which cause irreversible precipitation. The protein solution was centrifuged for 20 min in a Beckman Airfuge at 87,000 × g to remove insusable material. 15–20 ml of the supernatant were equilibrated by sitting drop vapor diffusion with 0.75 × NaNO3 dissolved in buffer. Crystals up to 0.8 × 1.0 × 1.3 mm3 grew within 1–2 days.

Data Collection and Processing—Three data sets were collected from three crystals. Two of lower resolution (up to 1.8 Å) were measured on a CAD-4 diffractometer, installed on a rotating anode x-ray generator FR 571 (Enraf-Norius, DelR) with a copper anode and nickel filter (45 kV, 99 mA, focus of 0.3 × 3 mm2, λ = 1.5418 Å, θ=2θ-scan mode). The orientation matrix was determined from the angle settings of 25 reflections, and 4 reference reflections were measured every 45 min to correct for crystal decay, which did not exceed 11% within 170 h of data collection. The data were corrected for Lorentz and polarization effects. A high resolution data set (3.5–1.4 Å) was measured with a FAST television area detector (Enraf-Norius, Delft) installed at station 9.6 of the synchrotron radiation source in Daresbury, United Kingdom, with a crystal-to-detector distance of 140 mm, a 2θ setting of 25°, and 0.1° rotation/frame over a total range of 60°. The data were processed with the MADNESS package (9) modified for synchrotron radiation (10).

The data were scaled and merged using ROTAVATA/AGROVATA of the CCP4 suite, yielding a Rmerge = 0.147 based on intensities >1σ (0.991 for I > σI), followed by treatment of weak reflections with TRUNCATE (11). The final data set contains 33854 unique θ=2θs (5% complete) within 10–1.5 Å (78% complete).

Refinement—The coordinates of the Ca2+-free proteinase K at 2.2 Å (8), without waters, served as a model in the refinement in the range 10–2.2 Å using the restrained reciprocal space refinement program TART (12). The initial crystallographic R-factor of 0.25 converged at 0.20 for all data up to 1.5 Å (0.18 for F > 3σ(F)), for 2029 protein atoms, one Na+, and 194 water molecules. 292 – Fc and 292 – F, maps were calculated with CCP4 programs and inspected with FRODO (13), and the model was improved manually. Poorly defined side chains were located in omit maps as described in (14). No cut in the main chain electron density was found, and all side chains could be traced, except Gly123, for which only density up to C, was found. The O2 of Ser35, Ser218, and Ser341 are 2-fold disordered with occupancies of 0.5. In a last cycle of refinement, the occupancies of the water molecules were refined with all other parameters held constant, and only those waters with occupancies above 0.5 were retained. The atomic coordinates and structure factors are deposited with the Brookhaven Protein Data Bank, entry number 2PKC.

The refined structures of native and Ca2+-free proteinase K were superimposed by a least squares fit using only the 1849 "isomorphous" atoms, i.e. corresponding atoms for which the distance after superposition was shorter than the mean coordinate error (0.15 Å) as estimated from the Luzzati plot (15). Each solvent molecule in one structure was compared with each solvent molecule of the other superimposed struc-
and 0.8 Å, respectively. The groove between the β-strand Asn197–Leu201 (βII8) and the loop Ser134–Pro168 (bridged by Ca2 in the native structure) is now widened because Ca2+ does not tether the carboxyl and amino termini anymore (Fig. 2). These changes are confined locally and do not extend to other parts of the protein. The temperature factors of most amino acids of either Ca2+ binding site increased upon removal of Ca2+, accompanied by changes in their backbone torsion angles (data not shown).

**Changes in Conformation**—The conformational differences between the structures of the native and the Ca2+-free proteinase K are only minute. Close inspection reveals that some segments of the polypeptide have shifted in a concerted manner upon removal of Ca2+, with major secondary structure elements not changing significantly while turns and loops exhibit the largest shifts. The root mean square distance between the 2014 common protein atoms is 0.5 Å, i.e. 0.2 Å for the 1116 main chain atoms, and 0.8 Å for the 898 side chain atoms; 180 protein atoms moved by more than 0.4 Å, and 105 main chain atoms moved by more than the most probable error of coordinates of both structures, 0.2 Å, as determined by the Luzzati plot (not shown).

The overall movement is best interpreted as two parts of the enzyme approaching each other upon removal of Ca2+ (Fig. 2). Starting from the Ca 1 binding site (loop Pro112–Val117), the motion of the “first part” is transmitted via β-strand βII2 (Asp186–Ser191) and βIII1 (Gly134–Tyr139), this is part of the recognition site and is twisted in the present structure), to helix α4 (Ser186–Val191). This acts as a lever, transducing the shift to βII5 (Met154–Ala159) and βII4 (Gly106–Leu111), which twists turn t10 (Asn125–Lys126), thereby conveying the movement to the “second part”, helix α3 (Gln152–Arg157), α3 again acts as a lever on the sequence Asp59–Gly105, which is also part of the recognition site and is twisted even more than Gly134–Tyr138. This conveys with a reduced solvent accessibility of the residues around Gly105.

Through the motion of the second part, loop Thr104–Gly108 shifts closer toward the recognition site. This is accompanied by the largest shifts of main chain atoms and highest B-factors outside the Ca2+ binding sites, transmitted by the central β-sheets in the core of proteinase K. This conrotarotating movement involves a “hinge” region (Ile215–Arg221) at the opposite side of the molecule (Fig. 2) where only subtle shifts of protein atoms occur.

**Active Site**—The geometry and the hydrogen bonding pattern (Table I) of the catalytic site (Asp58–His115, Ser224) did not change since these residues are located on rigid secondary structure elements. Also, the water molecule in the “oxyanion hole” (Wat335 in the native, and Wat262 in the Ca2+-free protease, distance 0.3 Å) is well defined and in the same position in both structures.

To examine how a substrate would fit into the recognition site of the Ca2+-free proteinase K, the coordinates of the complex between proteinase K and a peptide inhibitor (6) were superimposed with the present structure (root mean square distance, 0.5 Å for all the common 2014 atoms and 0.2 Å for the 1116 main chain atoms). Proteinase K recognizes its substrate by hydrogen bonding involving the backbone of the segments Ser134–Tyr104 and Ser132–Gly139 (6). Since the main chain atoms of Gly106–Tyr145 are shifted away from the inhibitor in the Ca2+-free structure by an average of 0.6 Å (maximum 0.9 Å at Gly106), the hydrogen bonds to the substrate are lengthened and consequently weakened. Upon substrate binding, an induced fit may occur, because the recognition site is flexible as indicated by the relatively high B-factors. Two waters (Wat197 and Wat198), which are not present in the native structure, have to be removed prior to binding of substrate in the Ca2+-free structure.
FIG. 2. Stereo plot of Ca$^{2+}$-free protease K. Helices are shown as ribbons, strands as arrows, and all other segments as coils. The catalytic triad (Asp$^{22}$, His$^{65}$, Ser$^{215}$) is drawn in ball-and-stick mode. Black arrows denote the contrarotating movements that take place due to the removal of Ca$^{2+}$ (see text). Elements of secondary structure that play a role in transmitting the long range movements are denoted or indicated by their residue number. Drawn with MOLSCRIPT (20).

**Solvent Structure**—In the $F_o - F_c$ map of the Ca$^{2+}$-free protease K, 193 peaks were interpreted as water molecules, and one was interpreted as Na$^+$ due to its coordination geometry (Fig. 1), while in the native crystal structure, 178 waters were found (4). 98 solvent molecules are conserved, and 96 appear only in the Ca$^{2+}$-free structure ("new" waters), while 82 are only found in the native enzyme ("lost" waters). Generally, the conserved positions are closer to the protein surface, and are coordinated more tightly than the others (Fig. 3), and six structurally important waters are buried inside the protein (with $<$5% accessibility: Wat$^{330}$, Wat$^{356}$, Wat$^{357}$, Wat$^{358}$, Wat$^{359}$, Wat$^{431}$). Around the low affinity binding site, Ca 2, several waters changed position. The change is due to an increase in solvent accessibility of several of these amino acids and is associated with the opening of the structure at this site (see above, Fig. 2).

The 2-fold disordered O$_i$ of three serines (15, 219, 247) are linked via water bridges to other residues that have changed significantly in position. These O$_i$ were not found disordered in the native protease K (Fig. 1), but, due to the substitution of Na$^+$ for Ca$^{2+}$, there is little positional change of the surrounding protein atoms (see above).

Around the low affinity binding site, Ca 2, several waters changed position. The change is due to an increase in solvent accessibility of several of these amino acids and is associated with the opening of the structure at this site (see above, Fig. 2).

The 2-fold disordered O$_i$ of three serines (15, 219, 247) are linked via water bridges to other residues that have changed significantly in position. These O$_i$ were not found disordered in the native protease K. Between the carboxyl termini of a4, a6, and turn t14 (part of the Ca 1 site), 13 waters are lost, while the largest group of new waters is found in the hinge region (Ile$^{212}$-Arg$^{218}$).

**Enzyme Kinetics and Thermal Stability**—To examine the influence of calcium on the proteolytic activity, both forms of protease K were assayed as described under "Materials and Methods." Table II lists the results.

While there is a strong dependence of the catalytic activity on pH for both the native and the Ca$^{2+}$-free enzyme with an optimum at pH 8-10, there is no difference with respect to either the affinity for the substrate (K$_m$) or the turnover number (k$_{cat}$). Also, no difference in fluorescence spectra between both forms of protease K can be observed, even after incubation up to 24 h at 23 °C (data not shown). These findings differ from the previous study in which an 80% decrease in activity 6 h after removal of Ca$^{2+}$ was reported (7). We explain this observation with the finding that Ca$^{2+}$-free protease K tends to precipitate irreversibly (see "Crystallization" under "Materials and Methods"), leading to a much reduced effective concentration in the assay.

The lower transition temperatures, T$_{m}$, obtained for the Ca$^{2+}$-free protease upon thermal denaturation (Table II) show a significant destabilization when Ca$^{2+}$ is removed. This is explained by the x-ray structure, which shows that Ca$^{2+}$ in both binding sites stabilizes the protein by bridging different parts of the tertiary structure.

**DISCUSSION**

Proteinase K binds two calciums, one strongly (Ca 1) and one weakly (Ca 2). Removing these ions invokes very subtle but concerted conformational changes that extend over 20 Å from the Ca 1 binding site via the peptide backbone to the substrate recognition site. These movements mostly affect loops and turns, while helices and central $\beta$-sheets are structurally invariant and transmit the shift. Removal of Ca 2, which tethers the amino and carboxyl termini, results in above average but locally confined geometrical changes. As an overall effect, the protein structure becomes more open, which is reflected by an increase in solvent accessibility of several residues (data not shown).

The difference in conformation is accompanied and probably stabilized by a change in local water structure, especially at the Ca 2 binding site, and in the hinge region. All of these structural differences between the crystal structures of Ca$^{2+}$-free and native protease K do not result in a significant reduction of the enzymatic activity. Therefore, we assume that the major role of Ca$^{2+}$ is to stabilize the native conformation as commonly observed in extracellular proteins (18). Studies on thermolysin (19) showed that removing calcium by EDTA causes the structure to relax and allows flexible parts to be more susceptible to proteolytic attack. One such structural element in proteinase K could be the loop Thr$^{35}$-Gly$^{68}$ (cf. Fig. 2), which exhibits one of the largest geometrical differences and highest B-factors in the Ca$^{2+}$-free structure. If an autolytic peptide cleavage would take place in this loop, it should displace the adjacent His$^{65}$ of the active site. However, this is not observed in the crystal structure as the main chain electron density is continuous and does not disclose any peptide cleavage. We assume that the expected
conformational changes associated with such a cut would give rise to a less well determined tertiary structure and that, consequently, molecules with a cut would not pack in an x-ray structure. This is supported by the finding that crystals of Ca²⁺-free proteinase K either grow within 1–2 days or else no crystals form. Also, solutions of the enzyme tend to precipitate irreversibly when under the influence of EDTA (see "Materials and Methods"), which was not observed if the solution contains Ca²⁺.

Acknowledgments—We thank M. Z. Papiz and P. J. Rizkallah for valuable advice and helpful discussions. The use of synchrotron beam time and financial support during data collection at Daresbury are gratefully acknowledged.

REFERENCES