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Crystal structure of an alkaline protease from *Bacillus alcalophilus* at 2.4 Å resolution

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The crystal structure of an alkaline protease from *Bacillus alcalophilus* has been determined by X-ray diffraction at 2.4 Å resolution. The enzyme crystallizes in space group $P2_12_12_1$ with lattice constants a = 53.7, b = 61.6, c = 75.9 Å. The structure was solved by molecular replacement using the structure of subtilisin Carlsberg as search model. Refinement using molecular dynamics and restrained least squares methods results in a crystallographic R-factor of 0.185. The tertiary structure is very similar to that of subtilisin Carlsberg. The greatest structural differences occur in loops at the surface of the protein.

Crystallization; Crystal structure; Alkaline protease; *Bacillus alcalophilus*

1. INTRODUCTION

Subtilisins (EC 3.4.21.14) are a class of extracellular endo-peptidases produced by a varity of *Bacillus* species [1]. These enzymes belong to the group of serine proteases with a mechanism of action identical to the trypsin-like mammalian serine proteases, but they are evolutionary and structurally distinct [2]. The enzymes from *Bacillus amyloliquefaciens* and *Bacillus subtilis* have been sequenced [3,4] and the genes coding for these subtilisins have been cloned [5-7]. The three-dimensional structures of the subtilisins from *B. amyloliquefaciens* have been determined previously (subtilisin BPN' [8], subtilisin Novo [9], subtilisin BAS [10]). For subtilisin Carlsberg from *B. subtilis* the crystal structure of the native enzyme [11] and the complex formed with the inhibitor eglin C [12] were reported. Because of extensive enzymatic as well as X-ray crystallographic studies on subtilisin this enzyme has provided an ideal model system for the rational alteration of enzyme properties using site-directed mutagenesis [13]. We obtained crystals of an alkaline protease from *Bacillus alcalophilus* which shows 59% homology and 3 deletions with respect to the amino acid sequence of subtilisin Carlsberg. Due to these differences it seemed interesting to obtain the structure of this protease. This paper describes the crystallization, structure determination and refinement of the alkaline protease from *B. alcalophilus* at 2.4 Å and compares the structure to that of subtilisin Carlsberg [12].

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2. MATERIALS AND METHODS

2.1. *Crystallization*

Alkaline protease from *Bacillus alcalophilus* (Opticlean) was a gift from Kali-Chemie (Hannover, FRG). Crystals of the protease inhibited by phenylmethylsulfonyl fluoride (PMSF) were grown using the hanging drop method. Samples (5 μl) containing 10 mg/ml protein were mixed with 5 μl of 1.0-1.2 M ammonium sulfate in 50 mM ammonium acetate buffer, pH 5.6-6.0 and were vapor equilibrated against the same buffers respectively. Crystals belong to the orthorhombic space group $P2_12_12_1$ with unit cell constants of a = 53.7, b = 61.6, c = 75.9 Å. The asymmetric unit contains one protein molecule with a molecular mass of 26 700.

2.2. *Structure determination and refinement*

X-ray diffraction data were measured to a resolution of 2.4 Å using a Xentronics area detector mounted on a Rigaku rotating anode X-ray generator (50 kV, 100 mA) with monochromatized CuKα radiation. The data were processed using the XENGEN program package [14]. Phases for the structure factors were determined by molecular replacement [15] using as a model the coordinates of subtilisin Carlsberg refined to 1.2 Å resolution [12] (1CSE; deposited in the Brookhaven Protein Data Bank [16]). A general search was performed with 6-8 Å data in 5° intervals. Peak positions were refined with 6-8 Å and 5-8 Å data in 2° and 1° intervals. A molecular radius of R = 25 Å was used. The rotation matrix corresponding to the highest peak in this rotation function map was applied to the model and a translation search was performed [17]. The relevant sections of the translation function showed a single high peak yielding a unique position for the molecule. The initial model of the protease was refined using the rigid body refinement program CORELS [18]. Starting with 6-10 Å data this procedure reduced the R-factor from 0.565 to 0.403 and improved the correlation coefficient from 0.42 to 0.55. Further refinement was performed by iteration of the restraint least squares calculation PROLSQ [19] and sessions of manual correction of the resulting coordinates by inspection of 2Fo-Fc difference Fourier maps using the FRODO software package [20] on an Evans and Sutherland PS 300 interactive graphics system. The initial resolution of 3-8 Å was increased during refinement to 2.4-8 Å. A molecular dynamics run with simulated annealing using the program XPLOR [21] reduced the R-factor from 0.37 to 0.26. The final refinement consisting of several

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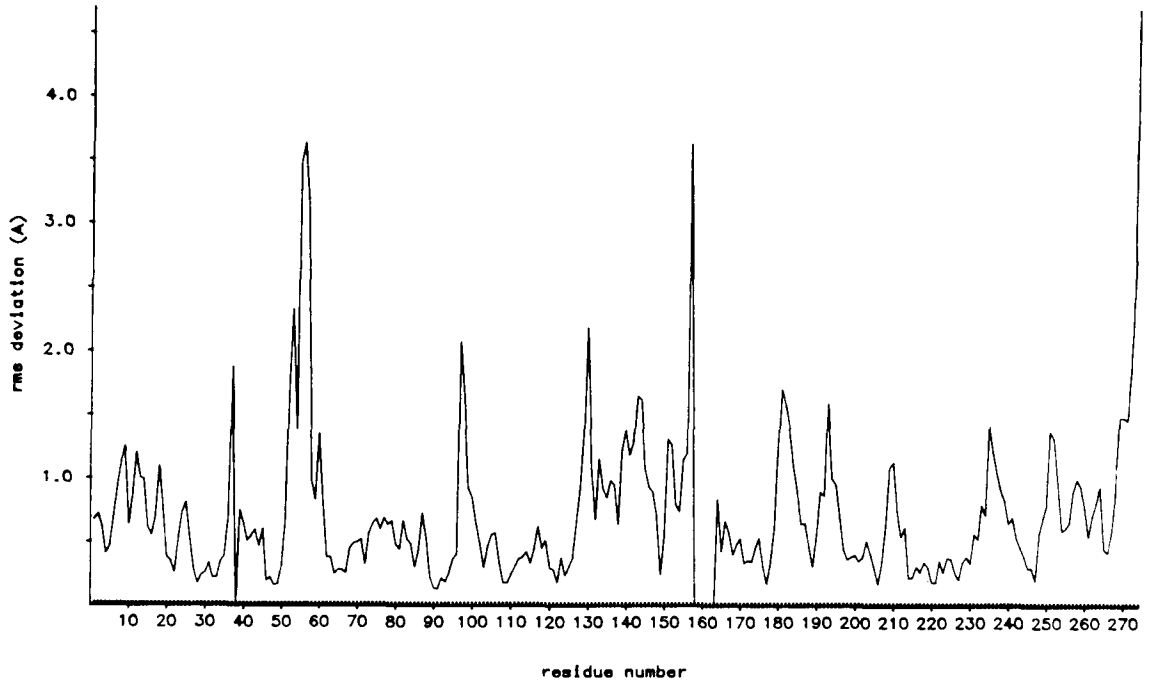

Fig. 1. Comparison of main chain positions of alkaline protease from *B. alcalophilus* with corresponding positions of subtilisin Carlsberg.

cycles of PROLSQ and manual rebuilding reduced the R-factor to 0.185 for all observed data from 8 to 2.4 Å. During the final stages of the refinement temperature factors were introduced for side chain and main chain atoms for each residue. 129 solvent molecules and 2 cations were located. The root-mean-square (rms) deviations of the model from ideal geometry are: 0.014 Å for the covalent bond distances, 0.047 Å for the interbond angle distances, 0.051 Å from the planar groups and 1.8 Å for the peptide bond torsional angles.

3. RESULTS AND DISCUSSION

Subtilisin is a globular protein belonging to the type B α/β proteins with a central β-sheet and helices running antiparallel to the strands of the sheet [22]. Detailed descriptions of the structure of subtilisin were

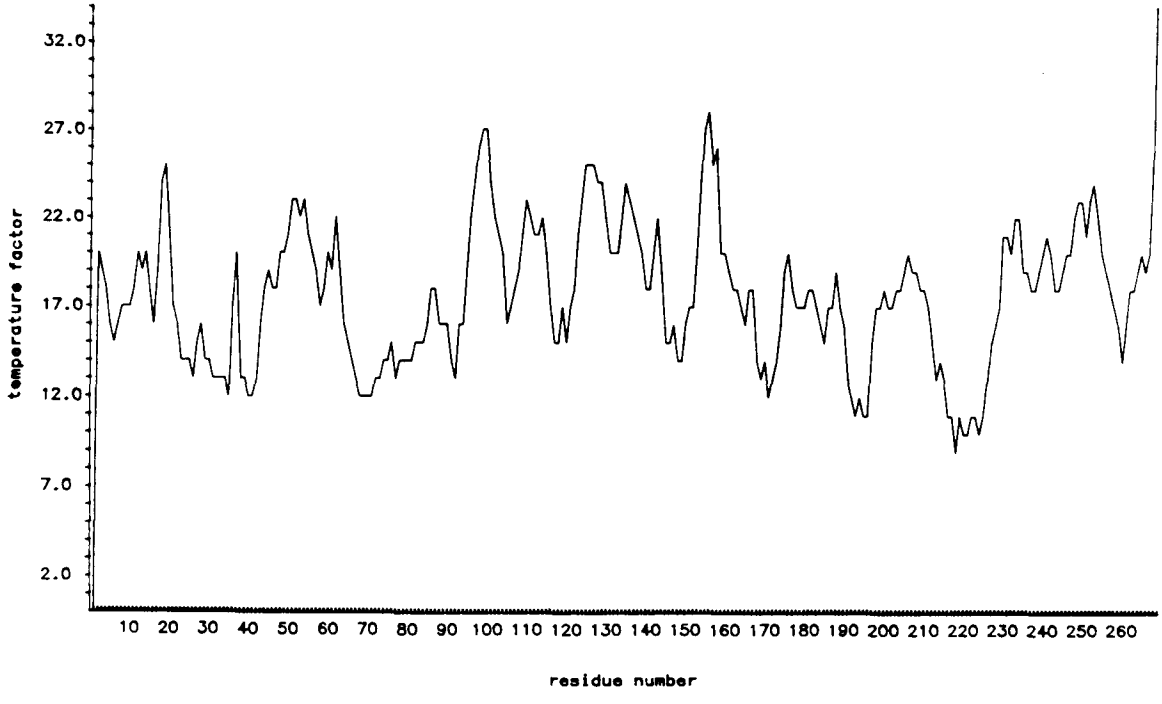

Fig. 2. Variation of the mean temperature B-factor for main chain atoms plotted versus residue number.

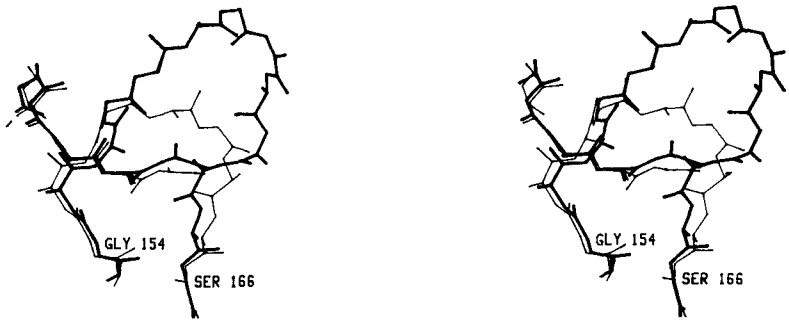

Fig. 3. Polypeptide chain from Gly154 to Ser 166 of *B. alcalophilus* protease (thin connections) superimposed with equivalent residues of subtilisin Carlsberg (thick connections).

published previously [8,12]. The general structure of the alkaline protease from *B. alcalophilus* is very similar to the structure of subtilisin Carlsberg [12]. Fig. 1 shows a plot of the variation in the positions of main chain atoms between *B. alcalophilus* protease and subtilisin Carlsberg. The root-mean-square deviation of corresponding main chain positions between *B. alcalophilus* protease and subtilisin Carlsberg is 0.94 Å. The figure shows several regions of structural differences. A general correspondence between the magnitude of the observed main chain B-factor (Fig. 2) for a given residue in alkaline protease from *B. alcalophilus* and the difference in main-chain coordinates between *B. alcalophilus* protease and subtilisin Carlsberg illustrates that higher deviations mostly occur in flexible loops at the surface of the protein. Some of these regions with structural differences are regions where deletions in the sequence of *B. alcalophilus* protease are tolerated without disturbance of structural or functional units of the protein. Compared with subtilisin Carlsberg the alkaline protease from *B. alcalophilus* contains deletions in position 38, 159 and 162–164. All deletions occur at the surface of the protease. The deletion in position 38 is compensated for by a shift of the preceding polypeptide chain. The deletion of four residues (159, 162–164) in the external loop 153–166 leads to a strong shortening of this loop without affecting residues 153–156, 165 and 166 participating in the formation of the P1-binding pocket (Fig. 3).

The catalytic triad of subtilisins is composed of residues Asp32, His64 and Ser221. Compared to subtilisin Carlsberg the active site in *B. alcalophilus* protease shows a similar arrangement of the backbone (rms deviation: 0.26 Å) and the side chains of Asp32 and Ser221. The side chain of His64 is rotated to an orientation perpendicular (rms deviation: 115 Å) to the carboxyl group of Asp32 prohibiting the usually observed hydrogen bonding (distances His64Nδ1 to Asp32Oδ1 and Oδ2 3.7 Å and 3.6 Å; His64Nδ2 to Asp32Oδ1 and Oδ2 3.4 Å and 3.3 Å). A distance of 3.05 Å between His64Nϵ2 and Ser125O seems to indicate a weak hydrogen bond between these residues. An additional weak electron density adjacent to Ser221Oγ might represent the sulfonyl part of the covalently bound inhibitor; however, no electron density could be observed for the aromatic part of PMS.

The *cis*-peptide bond between Tyr167 and Pro168 found in the reverse turn Ser166–Ala169 in subtilisin Carlsberg [11,12] is conserved in the described alkaline protease, whereas the second *cis*-peptide bond between Pro210 and Thr211 reported for the highly refined structures of subtilisin Carlsberg [12,23] is not visible. Co-ordinates will be deposited with the Brookhaven Data Base.

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