Increasing the thermostability of the neutral proteinase of *Bacillus stearothermophilus* by improvement of internal hydrogen-bonding

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In an attempt to increase the thermostability of the neutral proteinase of *Bacillus stearothermophilus* the buried Ala-170 was replaced by serine. Molecular-dynamics simulations showed that Ser-170 stabilizes the enzyme by formation of an internal hydrogen bond. In addition, the hydroxy group of Ser-170 could contribute to stability by filling an internal cavity. After the introduction of the mutation, using site-directed-mutagenesis techniques, an increase in stability of 0.7 ± 0.1 °C was obtained.

INTRODUCTION

Bacillus neutral proteinases (EC 3.4.24.4) are a group of well-characterized homologous endopeptidases sharing several properties, such as binding of Zn^{2+} and Ca^{2+} ions, inhibition by chelating agents, similar pH optima and a molecular mass of 32–35 kDa ([1,2] and references therein). The three-dimensional structures of the neutral proteinases of *Bacillus thermoproteolyticus* [3] (thermolysin) and *Bacillus cereus* [4] have been solved by X-ray crystallography. These structures have been used to build models of the neutral proteinases of *Bacillus subtilis* [2,5] and *Bacillus stearothermophilus* [2]. Neutral proteinases from different *Bacillus* species exhibit large differences in thermostability [6,7] and have therefore been used for studying structure–stability relationships [2,6,8,9].

Several studies have shown that mutational strategies used for increasing the stability of non-proteolytic enzymes by decreasing reversible unfolding [10,11] are also applicable to the stabilization of neutral proteinases, despite the occurrence of autolysis in the latter class of enzymes [2,6,9,12]. In one of these studies the neutral proteinase of *B. stearothermophilus* was stabilized by the mutation of Asn-241 to Leu [9]. This mutation resulted in the removal of an unsatisfied hydrogen-bond acceptor (Asn-241-O δ) from the interior of the folded protein. Obviously, an alternative to this approach would be the introduction of a hydrogen-bond donor capable of hydrogen-bonding with Asn-241-O δ . Inspection of the model of *B. stearothermophilus* neutral proteinase suggested that an Ala to Ser mutation at position 170 might be feasible for this purpose. The present paper describes the structural analysis of the Ala-170 to Ser mutation and the construction and characterization of the mutant protein.

MATERIALS AND METHODS

Model building and mutant prediction

The model of the *B. stearothermophilus* neutral proteinase was built on the basis of the X-ray structure of thermolysin [3] as described previously [2]. The model was studied by using the program WHAT IF [13] on an Evans and Sutherland PS390

interactive graphics system and an ESV 10 workstation. The automatic mutant prediction module in WHAT IF (G. Vriend, unpublished work) is based on a statistical analysis of local contact patterns as observed in a database containing 60 highly refined non-homologous protein structures. Running this module on the model of the neutral proteinase of *B. stearothermophilus* suggested that the Ala-170 to Ser mutation would be beneficial in terms of local contacts. Initial modelling of the mutant structure was performed by positioning the Ser-170 side chain in the most preferred rotamer attainable without introduction of unfavourable van der Waals overlaps. Preferred rotamers were determined by searching a protein structure database (206 proteins) for best-matching seven-residue fragments with the residue of interest in the middle position, using an α -carbon distance geometry algorithm similar to that of Jones & Thirup [14]. Selected fragments were superimposed in such a way that the α -carbons of the middle residues were exactly matched.

Energy minimizations and molecular dynamics were performed with GROMOS [15]. Restraints were placed on the α -carbon positions, such that they were kept within 0.025 nm (0.25 Å) of the original positions. These procedures were carried out as though the molecule was at 300 K and *in vacuo*, using the appropriate atomic partial charges incorporated in GROMOS. Since all relevant residues are fully buried in the enzyme, errors resulting from the omission of solvent molecules were expected to be small. The WHAT IF interface to GROMOS was used for the analysis of molecular-dynamics trajectories.

Hydrogen-bond parameters [16] were (maximum values): donor–acceptor distance 0.35 nm (3.5 Å); hydrogen–acceptor distance 0.25 nm (2.5 Å); angular error at the hydrogen 60°; angular error at the acceptor 90°. Cavities were defined as described by Rashin *et al.* [17] by using a probe with a 0.1 nm (1 Å) radius.

Site-directed mutagenesis

The *B. stearothermophilus npr* gene [18] was cloned, expressed and subcloned for site-directed mutagenesis as described before [2]. Site-directed mutagenesis was performed using the pMa/c system [19] as described previously [2]. The following mutagenic oligonucleotide primer was used (the changed nucleotide is

Abbreviation used: T_{50} , temperature resulting in 50% loss of activity after 30 min incubation.

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Fig. 1. Stereo drawings of the environment of residue 170 in *B. stearrowthermophilus* neutral proteinase

Relevant residues and hydrogen bonds (dashed lines) are shown. The positions of hydrogen atoms involved in hydrogen bonds are indicated by thin solid lines. Geometry data on some of the hydrogen bonds are given in Table 1. (a) Wild-type structure; contour lines indicate the cavity. (b) Initial modelling of Ser-170; Ser rotamers occurring in the 20 best-matching fragments that were found in the protein structure database (see the Materials and methods section) are shown as dotted lines; the selected conformation for Ser-170 is shown as a thick line. (c) The start (thick lines) and final (thin lines) conformation in a 2 ps molecular-dynamics simulation of the Ser-170 mutant (see the text for details); thin dotted lines indicate the path followed by relevant atoms in steps of 20 fs.

indicated by an asterisk; a *Cla*I restriction site that was introduced to facilitate selection of the mutant is underlined):

The DNA sequence of the mutated gene fragment was determined by using the dideoxy chain termination method [20].

Characterization of neutral proteinases

Production, purification and SDS/PAGE of the neutral proteinases were performed as described earlier [2,21,22]. For the determination of thermostability, the enzyme [$0.1 \mu\text{M}$ in 20 mM-sodium acetate (pH 5.0)/5 mM- CaCl_2 /0.5% (v/v) propan-2-ol/62.5 mM-NaCl] was incubated at various temperatures, ranging from 62 to 71 °C, for 30 min. Subsequently, the residual proteinase activity was determined using a casein assay [23] and

expressed as percentage of the initial activity. Thermostability was defined as T_{50} , being the temperature resulting in 50% loss of activity after 30 min incubation. Mutational effects were expressed as dT_{50} , being the difference in T_{50} between the wild-type and the mutant enzyme. Because T_{50} measurements exhibited fluctuations up to 1 °C, a wild-type reference sample was included in each measurement of dT_{50} . For the determination of dT_{50} , five independent assays were performed, resulting in an s.d. of the order of 0.1 °C.

RESULTS

Structural analysis

Fig. 1(a) shows the local environment of Ala-170 in the wild-type enzyme. All residues shown in Fig. 1(a) are located in the interior of the protein and are not accessible to solvent molecules.

Table 1. Hydrogen bonds for Ser-170-OH

The initial (after extensive energy minimization and before molecular dynamics) and final (after molecular dynamics) model of the Ser-170 mutant are shown in Fig. 1(c). The final model was subjected to a short-energy minimization before hydrogen bonds were calculated. $d(D-A)$ is donor-acceptor distance and $d(H-A)$ is hydrogen-acceptor distance (results in parentheses are in Å). $\angle(D-H-A)$ and $\angle(H-A-X)$ indicate deviations from optimal angles (in degrees) at the hydrogen and the acceptor respectively [16].

Model	Distance (nm)		Deviations (°)	
	$d(D-A)$	$d(H-A)$	$\angle(D-H-A)$	$\angle(H-A-X)$
Initial				
Ser-170-OH-Ala-166-O	0.267 (2.67)	0.178 (1.78)	33.4	62.7
Ser-170-OH-Ser-237-O	0.313 (3.13)	0.216 (2.16)	17.9	69.0
Final				
Ser-170-OH-Ser-237-O	0.301 (3.01)	0.224 (2.24)	46.4	63.6
Ser-170-OH-Asn-241-O δ	0.269 (2.69)	0.175 (1.75)	23.7	20.3

Fig. 2. Representative thermostability curves of wild-type (●) and mutant (○) neutral proteinase

The Ala-170 side chain is near a cavity, which has a volume of approx. 1 nm^3 (10 Å^3) (Fig. 1a). Serine can be positioned in its most preferred rotamer at position 170 without the introduction of van der Waals overlap (Fig. 1b). In this position its hydroxy group fills a large part of the cavity indicated in Fig. 1(a). The hydroxy group of Ser-170 can act as a bifurcated hydrogen-bond donor for hydrogen bonds with Ala-166-O and Ser-237-O (Fig. 1b; Table 1). In both the wild-type and the mutant enzyme Ala-166-O and Ser-237-O also function as acceptor in hydrogen bonds with backbone amides of residues 170 and 241 respectively.

The initial model of the mutant enzyme was subjected to extensive energy minimization. Subsequent molecular-dynamics simulations (Fig. 1c) showed little mobility of the Ser-170 side chain. However, the Asn-241 side chain rapidly (within 1 ps) reoriented itself, permitting the formation of a high-quality hydrogen bond with Ser-170-OH as donor and Asn-241-O δ as acceptor (Fig. 1c; Table 1). During the simulation, Ser-170-OH continued to act as donor in a hydrogen bond with Ser-237-O. The geometry of the latter hydrogen bond was poor in comparison with the geometry of the Ser-170-OH-Asn-241-O δ hydrogen bond (Table 1). Other hydrogen bonds were not significantly influenced by the reorientation of the Asn-241 side chain, mainly because of a concomitant minor reorientation of Asp-173.

Production and characterization of the mutant neutral proteinase

After construction of the mutant *npr* gene encoding the Ala-170 $\rightarrow$ Ser mutant, the mutant enzyme was produced and purified. The production level and the yield of the purification procedure

were within the range routinely obtained for the wild-type enzyme. The purified wild-type and mutant enzymes exhibited similar specific activities towards casein (approximate error 10%), and their mobilities during SDS/PAGE were identical (results not shown). Fig. 2 shows an example of the thermostability curves obtained for wild-type and mutant neutral proteinases. The Ala-170 $\rightarrow$ Ser mutation increased the T_{50} by $0.7 \pm 0.1^\circ\text{C}$ (average value of five independent assays $\pm$ S.D.).

DISCUSSION

The rate of irreversible thermal inactivation of neutral proteinases at elevated temperatures is determined by the kinetics of local unfolding processes which render the enzyme susceptible towards autocatalytic cleavage [12]. Local unfolding occurs in a late phase of the global unfolding process, when the protein is near its transition state for complete unfolding [12,24–26]. Since stability is determined kinetically, proteinases can be stabilized by mutations which increase the activation energy of unfolding. Thus mutations should stabilize the folded state relative to the transition state of unfolding and not necessarily relative to the unfolded state.

As a result of the Ala-170 $\rightarrow$ Ser mutation, the folded enzyme is stabilized by the extra high-quality Ser-170-OH-Asn-241-O δ hydrogen bond. The favourable effect of partially satisfying the hydrogen-bonding potential of Asn-241-O δ is offset by the introduction of the partially unsatisfied hydroxy group of Ser-170, which acts only as donor but not as acceptor in the folded mutant enzyme. Thus the difference in the number of hydrogen bonds between the folded and unfolded enzyme is identical in the wild-type and mutant neutral proteinase. However, the quality of the hydrogen bonds between Ser-170-OH (acting as a donor) and water in the solvated transition state is expected to be less than that of the internal Ser-170-OH-Asn-241-O δ hydrogen bond in the folded mutant protein [27]. Thus the stabilizing effect of the mutation results mainly from the stabilization of the folded state relative to the transition state and not relative to the unfolded state.

Filling a cavity as result of the Ala-170 $\rightarrow$ Ser mutation also contributes to the increase in stability. Quantitative analysis on the basis of literature data shows that the effect of filling a cavity of 1 nm^3 (10 Å^3) [17] is probably small as compared with the hydrogen-bonding effect [28].

The stabilizing effect of the Ala-170 $\rightarrow$ Ser mutation is similar to that of Asn-241 $\rightarrow$ Leu. On the basis of the currently available data, it is impossible to judge whether this is coincidental or is due to a limit to the amount of stabilization that can be achieved by site-directed mutations in the 170,241 region (see ref. [29] for

a detailed discussion of such possible limits). The fact that the effects of mutations in the interior of the *B. stearrowthermophilus* neutral proteinase often lie between -1°C and $+1^{\circ}\text{C}$ ([30]; V. G. H. Eijssink, G. Vriend and G. Venema, unpublished work) suggests that the observed identity is coincidental.

The reliability of the procedures used to build the model of the *B. stearrowthermophilus* neutral proteinase has been analysed by building a model of the *B. cereus* neutral proteinase before its X-ray structure had become available [31]. A comparison of the model and the X-ray structure [4] showed that the root-mean-square atomic misplacement for all atoms was 0.12 nm (1.2 Å). For the interior parts of the enzyme, taking into account only residues having less than 1 nm² (10 Å²) solvent-exposed surface area, the root-mean-square atomic misplacement amounted to only 0.06 nm (0.6 Å). The neutral proteinase of *B. stearrowthermophilus* has a higher sequence identity with thermolysin than that of *B. cereus* (85% and 73% respectively). Thus the model of the *B. stearrowthermophilus* neutral proteinase is probably more accurate than that of the *B. cereus* neutral proteinase. In the environment of residue 170 the model is expected to be highly accurate, since all residues involved are not exposed to solvent and they are conserved between thermolysin and the *B. stearrowthermophilus* neutral proteinase.

The increasing number of successful mutations, designed by using models of neutral proteinases based on X-ray structures of homologous proteins, indicates that model building by homology is a viable technique for obtaining valuable structural information. Although very useful in this respect, sequence homologies can also be misleading: despite the fact that thermolabile neutral proteinases have serine and thermostable neutral proteinases have alanine at position 170, the Ala-170 → Ser mutation in the thermostable *B. stearrowthermophilus* enzyme was beneficial for thermostability.

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