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Improving the thermostability of the neutral protease of *Bacillus stearothermophilus* by replacing a buried asparagine by leucine

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Amino acids buried in the hydrophobic interior of a protein with polar side chain atoms, which are not involved in hydrogen bonding or electrostatic interactions, have an adverse effect on protein stability. Replacing such residues by hydrophobic ones may render a protein more stable. Asparagine 241, which is buried in the neutral protease of *Bacillus stearothermophilus*, was replaced by leucine by site-directed mutagenesis. This mutation increased the stability of the protein by 0.7 ± 0.1 degree.

Thermostability; Neutral protease; Buried asparagine; *Bacillus stearothermophilus*

1. INTRODUCTION

Several members of the bacterial genus *Bacillus* are known to secrete metallo-endopeptidases, called neutral proteases. These enzymes consist of approximately 310 amino acids, one zinc atom, and one or more calcium atoms. Bacilli show considerable differences in their maximum growth temperature and the thermostabilities of their neutral proteases differ accordingly [1–3]. The amino acid sequences of several thermolabile and thermostable neutral proteases are known [2]. The three-dimensional structures of the neutral proteases of *B. thermoproteolyticus* (thermolysin) [4] and of *B. cereus* [5] have been solved by X-ray crystallography. These structures have been used to build a model of the structure of the neutral protease of *Bacillus stearothermophilus* [6].

When considering protein thermostability many entropic and enthalpic contributions have to be taken into account. For hydrophobic residues buried in an apolar environment the sum of entropic and enthalpic contributions is generally more favourable in the folded than in the unfolded form of the protein [7]. The opposite is generally true for polar groups which are buried in an apolar environment and which do not participate in hydrogen bonding or electrostatic interactions [8,9]. Therefore a protein can be stabilized by replacing such polar groups by hydrophobic groups, provided that the replacement is not accompanied by the introduction of too many negative effects (e.g. strain [10]).

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After analysis of the model of the *B. stearothermophilus* neutral protease structure, the polar Asn-241 was selected to be replaced by the non-polar leucine, which has approximately the same size and shape as asparagine. Asn-241 is buried in the hydrophobic interior of the protein and one of its polar side chain atoms is not involved in hydrogen bonding. The Asn-241 → Leu mutation increased the stability of the *B. stearothermophilus* neutral protease by 0.7 ± 0.1 °C.

2. MATERIALS AND METHODS

2.1. *Model building and mutant prediction*

The model of the *B. stearothermophilus* neutral protease was built as described before [6]. The program WHATIF [11] was used to search this model for buried polar amino acids with unsatisfied hydrogen bond donors or acceptors, as described previously [12]. The same program was used to search the protein structure database for matching substructures in a way similar to that of Jones and Thirup [13]. Hydrogen bonds were defined according to Baker and Hubbard [14], using the following, rather relaxed, upper limits: donor–acceptor distance 3.75 Å; hydrogen–acceptor distance 2.75 Å; angular error at the hydrogen 75 degrees; angular error at the acceptor 120 degrees. Surface area calculations were performed as described before [6].

2.2. *Plasmids and strains*

The *B. stearothermophilus npr* gene [15] was cloned into the high copy number vector pGE501 [6]. The gene was expressed in the protease deficient *B. subtilis* strain DB117 [6]. From pGE501 suitable fragments were subcloned in the *E. coli* plasmid pMa/c for site-directed mutagenesis [16]. *E. coli* WK6 and WK6MutS [17] were used in site-directed mutagenesis procedures. All strains were grown on trypton-yeast medium, containing appropriate antibiotics.

2.3. *Site-directed mutagenesis*

Oligonucleotide primers were designed and synthesized as described before [6]. Mutagenesis was performed using the gapped duplex method of Stanssens et al. [16]. Possible mutant clones (usually 15–50% of the obtained transformants) were selected by restriction

Fig. 1. A ribbon representation of the model of the neutral protease of *B. stearothermophilus*. Backbone and side chain atoms of Asn-241 are represented by black dots.

analysis [6] and their *npr* gene fragment was sequenced [18]. For the production of mutant neutral proteases in *B. subtilis*, correctly mutated fragments were used to construct pGE501 derivatives containing an intact mutant *npr* gene.

2.4. *Characterization of neutral proteases*

Production, purification and SDS-PAGE of neutral proteases were performed as described previously [6,19]. For the determination of thermostability, purified enzyme was diluted to a concentration of 3×10^{-7} M in 20 mM NaAc, pH = 5.0, 5 mM $CaCl_2$, 0.5% isopropanol and 62.5 mM NaCl. The enzyme dilutions were incubated at various temperatures, ranging from 62 to 72°C, for 30 min. After incubation the residual protease activity was determined using a casein assay [20]. The residual activities were expressed as percentage of the activity left after incubation on ice for 30 min.

3. RESULTS

3.1. *Structure analysis*

Fig. 1 shows the location of Asn-241 in the neutral protease of *B. stearothermophilus*. Fig. 2 shows the local environment of Asn-241 in greater detail. Upon replacing Asn-241 by leucine its unsatisfied hydrogen bond acceptor Oδ is removed. Also, its hydrogen bond donor Nδ, which forms a hydrogen bond with Oδ1 of Asp-173 (Table I) is removed. Since Asp-173-Oδ1 also forms a hydrogen bond with the N_ε of Arg-206 (Table I), the removal of the hydrogen bond between Asn-241-Nδ and Asp-173-Oδ1 does not introduce an unsatisfied hydrogen bond donor or acceptor. Thus, the net effect of the Asn → Leu replacement on hydrogen bonding is a decrease, by one, of the number of buried unsatisfied hydrogen bond acceptors in the folded enzyme. A second effect of the Asn → Leu mutation is an increase of the amount of buried hydrophobic surface area in the folded enzyme by 120 Å^2.

Table I

Hydrogen bonds for Asp-173-Oδ1

	d(D,A)	d(H,A)	∢(D-H-A)	∢(H-A-X)
Hydrogen bond:				
Asp-173-Oδ1–Asn-241-Nδ	3.00	2.04	18.9	59.7
Asp-173-Oδ1–Arg-206-Nε	2.81	1.88	26.9	63.9

d(D,A) is donor-acceptor distance. d(H,A) is hydrogen-acceptor distance. ∢(D-H-A) and ∢(H-A-X) indicate deviations from optimal angles at the hydrogen and the acceptor, respectively (in degrees).

A leucine in the environment shown in Fig. 2 has two preferred rotamers. The most preferred one, based on an analysis of 154 non-homologous protein structures, has χ_1 and χ_2 angles of approximately −60 and −180 degrees, respectively (Fig. 3). If the leucine is placed in the model such that it maximally overlaps with the original asparagine residue, it acquires these optimal side chain torsion angles. In this position no Van der Waals overlaps exist between the leucine side chain and any other residue.

3.2. *Production of mutant neutral protease*

The mutant *npr* gene encoding the Asn-241 → Leu mutant was constructed and the mutant enzyme was produced and purified. The wild-type and mutant enzymes showed no differences with respect to production level, yield of the purification procedure and specific activity towards casein. SDS-PAGE analysis of purified wild-type and mutant enzyme showed no differences (Fig. 4).

3.3. *Thermostability*

Fig. 5 shows a typical example of the thermostability curves of the wild-type and the mutant neutral pro-

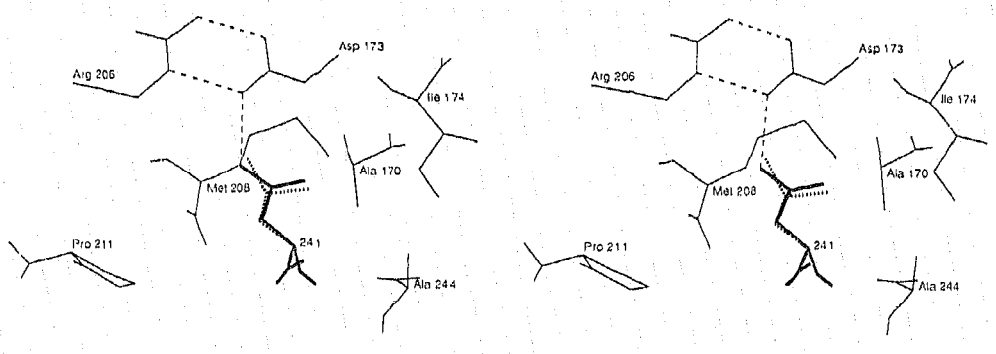

Fig. 2. Asparagine (thick solid line) and leucine (thick dotted line) at position 241 (stereo drawing). All residues having relevant interactions with the side chain of residue 241 are shown. Also Arg-206 is shown to illustrate its hydrogen bonding with Asp-173. Relevant hydrogen bonds are indicated by thin dotted lines. For clarity the backbone atoms of Asp-173 and Arg-206 were omitted.

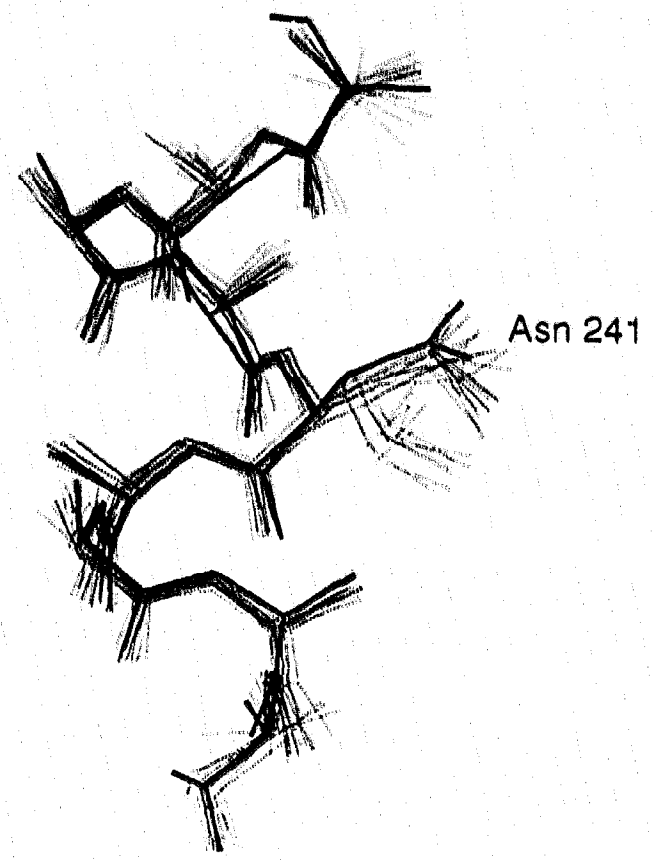

Fig. 3. Screening of a 154 protein database for matching substructures. The backbone of residues 237–255 and the side chain of Asn-241 are shown in thick lines. Dotted lines show 20 best matching substructures, having a leucine at position 241.

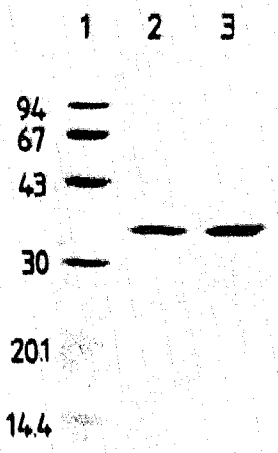

Fig. 4. SDS-PAGE analysis of wild-type and mutant neutral proteases. After electrophoresis gels were stained with Coomassie brilliant blue R-250. (Lane 1) Marker proteins (molecular weights are indicated in kDa); (lane 2) wild-type enzyme; (lane 3) Asn-241 → Leu mutant.

teases. Upon the Asn-241 → Leu mutation the survival temperature (the temperature which results in 50% loss of activity after 30 min of incubation) of the neutral protease is increased by 0.7 ± 0.1°C (average value of five independent assays).

4. DISCUSSION

It is commonly accepted that the thermostability of proteins cannot be attributed to one single determinant, but is the result of a variety of effects including hydrophobic interactions, ionic and hydrogen bonding, disulfide bridges and helix–dipole interactions. Several of these effects have been measured and/or calculated (for recent reviews see [7,21]).

An additional factor which co-determines the stability of proteases concerns their susceptibility to autocatalytic cleavage. Since several studies have indicated that reversible unfolding preceeds autocatalytic cleavage [22,23] it would seem that diminishing reversible unfolding might increase the thermostability of this type of enzymes. Therefore general approaches to increasing protein stability may also be applied succesfully to proteases [6]. In the present case this hypothesis was tested by replacing the polar asparagine-241 in the interior of the *B. stearothermophilus* neutral protease by a non-polar leucine.

The Asn → Leu mutation was expected to be beneficial for thermostability for two reasons. First, the difference in the number of hydrogen bonds between the folded and the unfolded protein is decreased and thus made more favourable [8,9]. Second, hydrophobic interactions are improved by increasing the amount of buried hydrophobic surface area in the folded protein [6,9,10]. The positive effects of the Asn → Leu mutation were not expected to be offset by the introduction

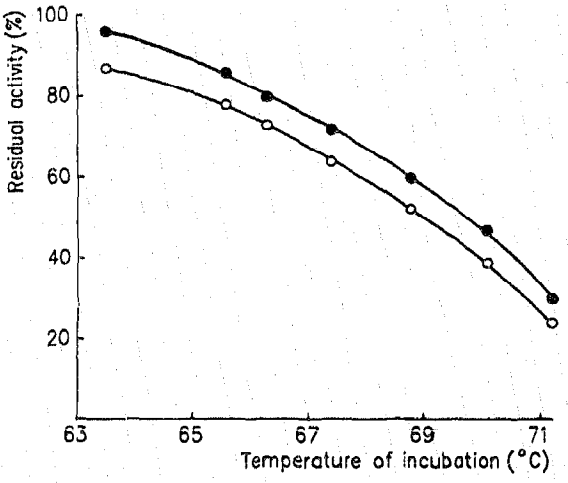

Fig. 5. Thermostability curves of wild-type (○) and mutant (●) *B. stearothermophilus* neutral protease.

of suboptimal torsion angles or Van der Waals overlaps. These inferences, derived from structural analyses of the wild-type and the mutant enzyme, were corroborated by showing that the thermostability of the mutant enzyme had increased.

In designing functionally active mutants, homologies with related proteins are often used for the identification of residues which might be changed and residues which should preferably remain unaltered. On the basis of homology studies Asn-241 would not have been selected for mutagenesis, since this residue is conserved in all *Bacillus* neutral proteases and it resides in a rather conserved region of the molecule. Nevertheless, its modification created an active enzyme with increased thermostability.

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