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Stabilization of *Bacillus stearothermophilus* neutral protease by introduction of prolines

F. Hardy[a], G. Vriend[b], O.R. Veltman[a], B. van der Vinne[a], G. Venema[a] and V.G.H. Eijsink[a,*]

[a]*Department of Genetics, Center for Biological Sciences, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands* and [b]*EMBL, Protein Design Group, Meyerhofstrasse 1, 6900 Heidelberg, Germany*

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The thermostability of neutral proteases has been shown to depend on autolysis which presumably occurs in flexible regions of the protein. In an attempt to rigidify such a region in the neutral protease of *Bacillus stearothermophilus*, residues in the solvent-exposed 63–69 loop were replaced by proline. The mutations caused large positive (Ser-65→Pro, Ala-69→Pro) or negative (Thr-63→Pro, Tyr-66→Pro) changes in thermostability, which were explained on the basis of molecular modelling of the mutant proteins. The data show that the introduction of prolines at carefully selected positions in the protein can be a powerful method for stabilization.

Thermostability; Neutral protease; Proline; Autolysis

1. INTRODUCTION

Bacillus neutral proteases (NPs) are homologous metalloendopeptidases with similar enzymatic characteristics [1]. Bacilli exhibit large differences in optimum growth temperature and, accordingly, their NPs differ considerably in temperature optimum and thermostability [2–4]. The amino acid sequences of several NPs are known [5–9] and the three-dimensional structures of the neutral proteases of *B. thermoproteolyticus* (thermolysin) and of *B. cereus* (NP-cer) have been solved by X-ray crystallography [10,11].

The factors that determine NP thermostability have been the subject of several studies [3,4,12–18]. At elevated temperatures NPs are irreversibly inactivated as a result of autolysis and it has been postulated that local unfolding processes preceding autolysis are the rate-limiting step in the thermal inactivation process [4,19]. It has been suggested that flexible surface-located regions, that relatively easily unfold to the extent that they become cleavable, are the main structural determinants of NP thermostability [4,17]. On the basis of the observation that the NP of *B. stearothermophilus* (NP-ste) is highly sensitive towards mutations at positions 63 and 69 [9,20], the surface-exposed 63–69 loop in this enzyme was proposed to be part of such a critical region. The 63–69 region was therefore selected as a target for site-directed mutations aimed at stabilizing NP-ste.

Correspondence address. G. Venema, Department of Genetics, Center for Biological Sciences, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands. Fax: (31) (50) 63 23 48.

**Present address.* Laboratory of Microbial Gene Technology, PO Box 5051, 1432 Ås, Norway.

Because prolines restrict the conformational freedom of the backbone of polypeptide chains [21], they are expected to contribute to protein stability by reducing the entropy of the unfolded state [22,23]. It was anticipated that the introduction of prolines would be a good stabilization strategy for proteases, since prolines might reduce local unfolding and the flexibility of the locally unfolded structures, thus reducing autolysis. To analyze the feasibility of this strategy several Xxx→Pro mutations were made in the 63–69 region of NP-ste.

2. MATERIALS AND METHODS

2.1. *Genetics*

Bacterial strains were grown on trypton-yeast medium, containing appropriate antibiotics. The *npr* gene of *B. stearothermophilus* was cloned, subcloned and expressed as described before [14,15]. Site- directed mutagenesis was performed as described previously [15], using the pMa/c gapped duplex system [24]. The nucleotide sequences of mutated gene fragments were verified using dideoxy DNA sequencing [25].

2.2. *Production and characterization of mutated enzymes*

Production, purification and subsequent characterization of enzymes were performed as described earlier [4,15]. The thermostability of mutated proteins at pH 5.3 was evaluated by the determination of T50, as described before [4]. T50 is the temperature at which 50% of the initial enzymatic activity remains active after a 30-min incubation period. Proteolytic activities were determined using casein as a substrate [26]. The thermostabilities of the mutant proteins were expressed as ΔT50, being the difference in T50 between the mutant and wild-type NP-ste.

2.3. *Structural analysis*

The high sequence homology between thermolysin [5] and NP-ste [7] (85% sequence identity) permitted the construction of a three-dimensional model for NP-ste expected to be sufficiently reliable to predict the effects of site-directed mutations [27,28]. The modelling procedures were described in detail elsewhere [15]. The 63–69 regions of NP-ste and thermolysin were expected to have highly similar structures. This idea was supported by inspection of the crystal structure

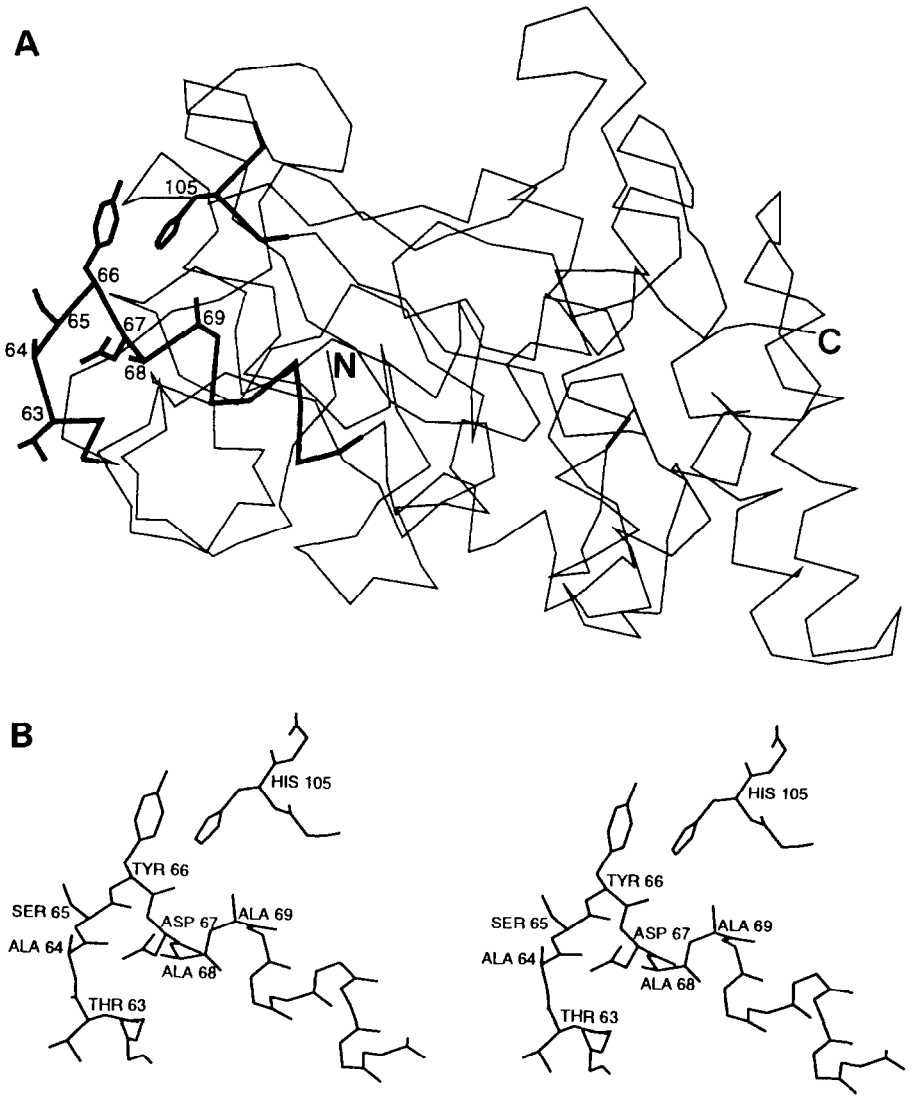

Fig. 1. Structure of the 63–69 region in the neutral protease of *B. stearothermophilus*. (A) Cα trace of the model of NP-ste, including the side chains of the residues mentioned in the text. These residues and the N-terminal (N) and C-terminal (C) residues are labeled. The regions drawn in thick lines are shown in detail in B (stereo picture).

of NP-cer [11]. The latter enzyme differs from thermolysin to a larger extent (73% sequence identity) than NP-ste, yet it contains the same fold as thermolysin in the 63–69 region [11,20] The relevant parts of the structure of NP-ste are shown in Fig. 1. Structure analyses, modelling of mutant enzymes, database searches and molecular graphics were done with the WHAT IF program [29]. Hydrogen bonds were defined according to Baker and Hubbard [30], using the following upper limits: donor–acceptor distance 3.50 Å; hydrogen–acceptor distance 2.50 Å; angular deviation at the hydrogen 60 degrees; angular deviation at the acceptor 90 degrees. Residues are numbered throughout the paper according to the sequence of thermolysin

3. RESULTS AND DISCUSSION

The contribution of a residue to the overall stability of a protein is the summation over a large number of positive and negative terms. The introduction of a proline will only have a stabilizing effect if its positive effect is larger than negative side effects, such as e.g. the introduction of conformational strain, or the disruption of favourable interactions that exist in the wild-type enzyme. The introduction of conformational strain in the backbone deserves special attention, since prolines have less freedom in their backbone than the other 19 amino acids. This is illustrated in Fig. 2, showing the limited variation in phi-psi combinations observed within the collection of all 2232 prolines in a non-redundant subset [31] of 241 proteins from the Brookhaven protein structure databank [32]. In contrast to all other amino acids, the backbone nitrogen of a proline does not have the capacity to act as a hydrogen bond donor. Therefore the introduction of prolines may have the unfavourable side effect of disrupting a hydrogen bond involving the backbone amide of the replaced residue.

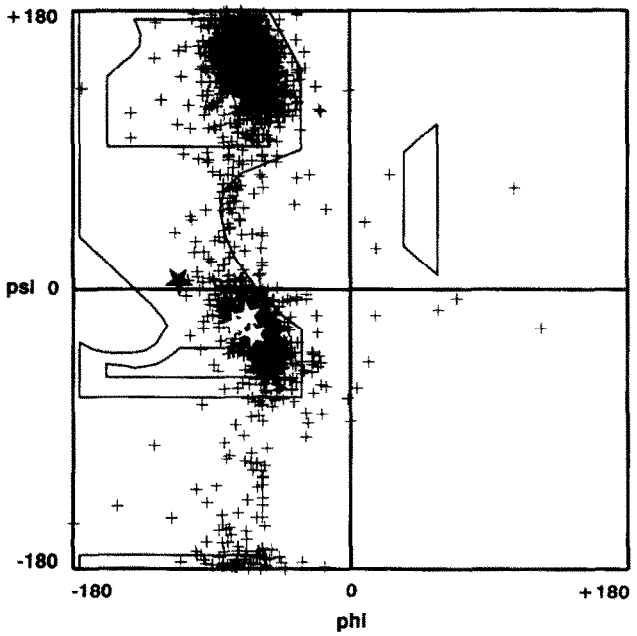

Fig. 2. Ramachandran plot for 2232 prolines. Asterisks indicate the phi-psi angles of the four mutated residues in wild-type NP-ste. The top left asterisk is Thr-63.

An analysis of the structure of the 63–69 region revealed two suitable candidates for substitution by proline: Ser-65 and Ala-69. The feasibility of the Ala-69→Pro mutation was indicated by the fact that it occurs in nature: thermolysin contains a proline at position 69. Both Ser-65 and Ala-69 have phi-psi angles that are favourable for proline (Fig. 2) and their backbone amides are not involved in intramolecular hydrogen bonds. The side chain of residue 65 is completely solvent-exposed and does not have significant interactions with neighbouring residues in wild-type NP-ste nor in the Pro-65 mutant. The side chain of residue 69 is only partly solvent-exposed. Its $C\beta$ is involved in several hydrophobic contacts which are similar in wild-type NP-ste and the Pro-69 mutant [20]. The introduction of a proline at position 69 in NP-ste seemed possible without introducing Van der Waals clashes. On the basis of these analyses it was expected that the presumed rigidifying effect of the Ser-65→Pro and the Ala-69→Pro mutations would not be offset by negative side effects.

Inspection of the model of NP-ste showed that prolines can not be introduced at the positions 64 and 68 without the introduction of major Van der Waals clashes (with the side chain of Asp-67 and the backbone of Ser-65, respectively). Asp-67 was not considered for replacement because it is supposed to contribute to thermostability by coordinating, via a water molecule, a bound calcium ion ([33], G. Vriend et al., unpublished results). Thr-63 seemed a reasonably suitable candidate for replacement, because neither the threonine in the wild-type, nor the proline in the mutant have important interactions with other residues, and the backbone amide of residue 63 is not involved in intramolecular hydrogen bonds. However, the backbone torsion angles of Thr-63 are not common for proline (Fig. 2). The solvent-exposed Tyr-66 has phi-psi angles that are perfect for proline (Fig. 2) and its backbone amide is not involved in intramolecular hydrogen bonds. However, the tyrosine side chain has several favourable contacts with other residues (most prominently with His-105; Fig. 1). These contacts would be lost upon the Tyr-66→Pro mutation.

We decided to construct a set of four mutants (Thr-63→Pro, Ser-65→Pro, Tyr-66→Pro, Ala-69→Pro), comprising two mutations likely to be stabilizing (Ser-65→Pro, Ala-69→Pro), and two mutations with unfavourable side effects of a rather different nature (backbone strain for Thr-63→Pro; loss of favourable interactions for Tyr-66→Pro). None of these mutations affected the specific activity towards casein or the electrophoretic mobility of NP-ste (results not shown). The effects on thermostability are shown in Table I and Fig. 3. The 'ideal' Ser-65→Pro and Ala-69→Pro mutations indeed stabilized NP-ste, by 4.2 °C and 5.5 °C, respectively. The two non-ideal mutations, Thr-63→Pro and Tyr-66→Pro, had clear destabilizing effects. Apparently, the anticipated negative side effects of these mutations are larger than the beneficial effect of rigidification.

Since the presence of a proline reduces the susceptibility of the two preceding peptide bonds towards cleavage by an NP [34–36], it is conceivable that the introduction of prolines affected the thermostability of NP-ste by a direct effect on the cleavability of the mutated chain segment. This is however highly unlikely. First, the sequence specificity of NPs is such that it is unlikely that the peptide bonds that are affected by the present Xxx→Pro mutations are major autolytic target sites. Only in case of the Thr-63→Pro mutation a direct inhibitory effect on cleavability could be envisaged, but this mutation actually destabilized the enzyme. Second, additional mutations at positions 65 and 69 have indicated no correlation between the effect on thermostability and the presumed effect on cleavability of the peptide bonds involved (F. Hardy and V.G.H. Eijsink, unpublished results).

The data presented here clearly show the power of

Table I

Thermostability of mutant *B. stearothermophilus* neutral proteases

Mutant	ΔT50 (°C)
Thr-63→Pro	−10.0
Ser-65→Pro	+4.2
Tyr-66→Pro	−16.0
Ala-69→Pro	+5.6

ΔT50 values were determined as described in section 2; they represent the average value of at least three independent assays; error margins were approximately −0.3°C. The T50 of wild-type NP-ste was 68.5°C.

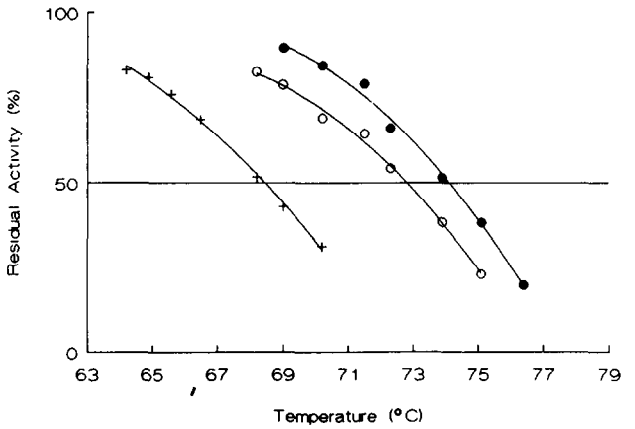

Fig. 3. Representative thermostability curves of wild-type *B stearothermophilus* neutral protease (+) and the Ser-65→Pro (○) and Ala-69→Pro (●) mutants. The curves show the relative residual proteolytic activity after a 30-min incubation period as function of the incubation temperature.

stabilizing an enzyme by introducing proline residues. However, the destabilizing effects observed for the mutations at positions 63 and 66 show that only a limited number of residues can be successfully replaced by proline. Careful analysis of the structural context of the mutated position is necessary, since the beneficial effect of introducing proline can very easily be offset by unfavourable side effects. The effect of the Thr-63→Pro mutation shows that the backbone torsion angles of the replaced residue are critical. The effect of the substitution at position 66 indicates the importance of (lost) contacts. In case no unfavourable side effects are to be expected (like for the Ser-65→Pro and Ala-69→Pro mutations), the introduction of prolines seems to be an effective stabilization strategy. This strategy might be particularly effective for rigidifying surface areas in proteases that are susceptible towards local unfolding and subsequent autolysis. It seems a valuable strategy for the protection of proteins in general against local unfolding and subsequent proteolytic attack.

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