Cavities in the hydrophobic core of the neutral protease of *Bacillus stearothermophilus* were analyzed using a three-dimensional model that was inferred from the crystal structure of thermolysin, the highly homologous neutral protease of *B. thermostreptolyticus* (85% sequence identity). Site-directed mutagenesis was used to fill some of these cavities, thereby improving hydrophobic packing in the protein interior. The mutations had small effects on the thermostability, even after drastic changes, such as Leu284→Trp and Met168→Trp. The effects on T50, the temperature at which 50% of the enzyme is irreversibly inactivated in 30 min, ranged from 0.0 to +0.4°C. These results can be explained by assuming that the mutations have positive and negative structural effects of approximately the same magnitude. Alternatively, it could be envisaged that the local unfolding steps, which render the enzyme susceptible towards autolysis and which are rate limiting in the process of thermal inactivation, are only slightly affected by alterations in the hydrophobic core.

Key words: Bacillus/cavity/hydrophobic/neutral protease/thermostability

**Materials and methods**

**Genes, plasmids and site-directed mutagenesis**

A 2.35 kb Ncol–BclI fragment from plasmid pNP22 (Fuji et al., 1983), containing the *B. stearothermophilus* npr gene, was subcloned in the BclI site of the high-copy number *B. subtilis* vector pT72 (Aoki et al., 1987), yielding pGE501 (Figure 3). For the subcloning procedure and for production of the enzyme *B. subtilis* strain DB117 (Eijsink et al., 1990) was used. For site-directed mutagenesis (Stanssens et al., 1989) suitable fragments of the npr gene were subcloned in the *E. coli* vector pMa/c. For the mutagenesis procedure *E. coli* strains WK6 and WK6mutS (Zell and Fritz, 1987) were used. After mutagenesis the DNA sequence of the mutated gene fragment was verified using the dideoxy chain termination method (Sanger et al., 1977).

**Table I. Oligonucleotides used for site-directed mutagenesis**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>M168W</td>
<td>5'-CGAAAATATCGGACCACGCTTCATTTG-3' (Styl)</td>
</tr>
<tr>
<td>A240V</td>
<td>5'-GCGAAGTACCGCACTTTTAATTTGATGCGCCG-3' (Asnl)</td>
</tr>
<tr>
<td>A241W</td>
<td>5'-GAGCAAGTACGCACTTTTAATTTGATGCGCCG-3' (Asnl)</td>
</tr>
<tr>
<td>L284W</td>
<td>5'-CGCAAGGCGGCGCCACTGGTCGAGG-3' (BssHII)</td>
</tr>
<tr>
<td>C288L</td>
<td>5'-CCGGCTTTGCCCACCGCGCAGG-3' (Styl)</td>
</tr>
<tr>
<td>C288R</td>
<td>5'-CGCTTGCCCACATCAGGCGCCAGCG-3' (PvuII)</td>
</tr>
</tbody>
</table>

*The wild-type sequence of the *B. stearothermophilus* npr gene has been described by Takagi et al. (1985). Nucleotide substitutions are shown in bold type face. Restriction sites, inserted to facilitate selection of mutant clones, are shown between brackets and underlined in the sequence.*
Subsequently complete genes, encoding mutant neutral proteases were reconstituted in *B. subtilis* DB117. Oligonucleotides used for site-directed mutagenesis are shown in Table I.

**Production, purification and characterization of neutral proteases**
Production, purification and SDS-PAGE of wild-type and mutant neutral proteases were performed as described previously (Van den Burg et al., 1989; Eijsink et al., 1990). After purification the enzymes were stored in elution buffer [20 mM sodium acetate, pH 5.0, 5 mM CaCl₂, 2.5 M NaCl, 20% (v/v) isopropanol, 0.03% (w/v) sodium azide] at -18°C. For the determination of thermostability, aliquots of diluted enzyme [0.1 μM in 20 mM sodium acetate, pH 5.0, 5 mM CaCl₂, 62.5 mM NaCl, 0.5% (v/v) isopropanol, 0.03% (w/v) sodium azide] were incubated for 30 min at various temperatures. Subsequently, residual activities were determined using a casein assay (Fujii et al., 1983). Residual activities were expressed relative to the initial activity. T50 is the temperature at which 50% of the initial activity was retained. The thermostability of the mutant enzymes is expressed as dB50, being the difference in T50 between the mutant and the wild-type enzyme.

**Model building and mutant prediction**
The alignment between thermolysin (316 residues) and Npr-ste (319 residues) has been described by Eijsink et al. (1990). Npr-ste has three extra residues inserted between residues 28 and 29 in thermolysin. In this paper all residues in Npr-ste are numbered according to the thermolysin amino acid sequence.

The model of Npr-ste, built on the basis of the structure of thermolysin (Holmes and Matthews, 1982), has been described elsewhere (Eijsink et al., 1990, 1992a). Molecular modelling was performed with the program WHAT IF (Vriend, 1990) using an Evans and Sutherland PS390 interactive graphics system and an ESV10 workstation. Cavities were identified with the fast surface detection method of Voorintholt et al. (1989), as implemented in WHAT IF (Vriend, 1990). This method gives similar results to the methods described by Conolly (1985) and Rashin et al. (1986). The grid spacing was 0.72 Å. The probe diameter was set at 1.5 Å in order to define as a cavity the space in which a covalently bound methyl group could be positioned. Such a methyl group would extend an existing side chain by ~1.5 Å. The volume of a cavity was defined as the volume enclosed by the surface traced out by the probe sphere. Van der Waals radii used were: S, 2.0 Å; N, 1.7 Å; C, 1.8 Å; O, 1.4 Å.

Possible conformations of the side chains of introduced residues were analyzed by searching a protein structure database (206 proteins) for the best-matching 7-residue fragments with the residue of interest in the middle position, using an alpha-carbon distance geometry algorithm similar to that of Jolles and Thirup (1986). The side chain of the mutant residue was positioned in the most preferred rotamer that was attainable without the introduction of unfavourable Van der Waals overlaps.

Subsequently, the mutant structures were subjected to energy minimization, followed by 2 ps molecular dynamics at 300 K to get out of high energy local minima, and a final, exhaustive energy minimization, using GROMOS (Van Gunsteren and Berendsen, 1987). During these procedures constraints were placed on the alpha-carbon positions to keep them within ~0.25 Å of the original positions. The procedures were carried out using the atomic partial charges incorporated in GROMOS for calculations in vacuo.
devoid of crystal waters and it was assumed that this also applies to the corresponding cavities in Npr-ste. In the model of Npr-ste the total volume of the cavities shown in Figure 2 is ~ 50 Å³.

To verify whether the energy minimization and molecular dynamics procedures that were used in the final stage of model building (Eijsink et al., 1990) could have introduced artifacts with respect to cavities in the Npr-ste model, the X-ray structure of thermolysin was subjected to the same procedures. Because of these procedures, several cavities in the thermolysin structure were changed, indicating that errors indeed occur and that modelled cavities may differ from reality. However, the cluster of cavities around Phe267 remained largely unaltered, showing that in this area the impact of energy minimization and molecular dynamics on the shape and size of cavities was small. On the basis of these observations and because of the fact that most residues around Phe267 are conserved between Npr-ste and thermolysin, it was assumed that the Npr-ste model was sufficiently accurate for adequate modelling of mutations designed to fill the cavities in this region.

Modelling studies suggested that several mutations at each of the five positions shown in Figures 1 and 2 could reduce the size of the cavities. Those mutations were selected that filled a cavity as much as possible, while keeping the number of unfavourable side effects (conformational strain; Van der Waals overlaps) at a minimum. In two cases it appeared that this might be achieved by introducing Trp residues, at positions 168 and 284. Figure 4(A–F), presenting details of the modelled structures of the mutant enzymes, suggests that most of the introduced hydrophobic side chains can be accommodated by the cavities without introducing Van der Waals overlaps. Important structural effects of the mutations are summarized in Table II.

### Mutant neutral proteases

Using oligonucleotide-directed mutagenesis, mutant npr genes were constructed, encoding the Npr-ste variants listed in Table II. The mutant enzymes were similar to the wild-type enzyme with respect to the level of production by B. subtilis DB117 and electrophoretic mobility during SDS–PAGE. After purification, the mutant and wild-type enzymes exhibited similar specific activities towards casein (data not shown). The thermostabilities of the mutant enzymes are indicated in Table II. The data in this table show that the effects of the mutations on thermostability varied from zero to slightly positive.

### Discussion

On the basis of model-building of the three-dimensional structure of Npr-ste and mutants thereof, it was expected that the cavity-filling mutations described above would improve the packing density and Van der Waals interactions in the hydrophobic core of this protein, thus increasing its thermostability. Indeed, some of the mutations increased the thermostability of Npr-ste, although their effect was small. Those substitutions that concerned the largest side chains tended to result in the most pronounced increase in thermostability of the enzyme.

To compensate for the uncertainties inherent to the model-building procedures we constructed six different mutations to fill the cavities. Remarkably, none of these mutations destabilized the protein, despite the model-building uncertainties and some obvious negative side effects (listed in Table II). A double mutant, containing Trp at positions 284 and 310 (Leu and Phe in wild-type Npr-ste, respectively) was only slightly destabilized (~0.4°C; V.G.H. Eijsink, O.R. Veltman and B.W. Dijkstra, unpublished observations). The difference in dT50 between the Leu288 and the Ile288 mutants was expected from a comparison of their respective models. As shown in Figure 5, both introduced side chains have Van der Waals overlaps with the side chain of Phe267, which bends away in the model building procedures. Bending is more pronounced in the case of the Leu288 mutant, which, accordingly, has a lower thermostability. Interestingly, Phe267 and Phe310 (Figure 4) have the second and third highest B-factors of all buried residues in the crystal structure of thermolysin (Holmes and Matthews, 1982), indicating that these residues have relatively high conformational freedom. Thus the movement of Phe267 that resulted from the mutations at position 288 is probably only slightly unfavourable, explaining why the Cys288-Leu and Cys288-Ile mutations exerted a stabilizing effect, despite the Van der Waals overlaps.

Site-directed mutagenesis experiments have supported the general view that the hydrophobic core of a protein represents a delicate balance between high packing density and the conformational strain needed to achieve this (Sandberg and Terwilliger, 1989, 1991; Karpusas et al., 1989). It has been shown that a protein can adjust the packing of its hydrophobic core to accommodate a great variety of mutations as long as hydrophobicity is preserved (Lim and Sauer, 1989). However, such mutations often introduce significant negative effects such
Van den Burg et al., 1991) and 69 (dT50 for Ala69Pro = +5.5°C; V.G.H.Eijsink, G.Vriend and G.Venema, unpublished results). Mutations at buried positions in Npr-ste had smaller stabilizing effects (in the range of +1.0°C; Eijsink et al., 1991b, 1992a; Vriend et al., 1991).

In order to discriminate between the two explanations for the insensitivity of Npr-ste towards cavity-filling mutations it would be desirable to study reversible global unfolding of the Npr-ste variants and thus measure the energetic effects of the mutations. However, such studies are complicated, if not impossible, because of the occurrence of autolysis (Dahlquist, 1976; Mitchinson and Wells, 1989; Eijsink et al., 1991a). Further insight might be gained by mutations that result in the removal of hydrophobic groups from the protein interior. Their effect would be stabilizing if the first explanation were valid, whereas thermostability would be largely unaffected if the second explanation held.

Rigorous structural analysis of the site-directed mutations is hampered by the lack of crystallographic data for both the wild-type and the mutant neutral proteases. However, previously described data concerning the model-building procedure (Eijsink et al., 1992a) and the engineering of neutral protease stability (Imanaka et al., 1986; Eijsink et al., 1990, 1991b, 1992a,c,d;
The hydrophobic core of neutral protease

Fig. 4. Modelled structures of the environment of mutated residues. (A) Met168Trp, (B) Ala240Val, (C) Ala241Val, (D) Leu284Trp, (E) Cys288Leu, (F) Cys288Ile. Important characteristics of these structures are summarized in Table II.

Fig. 5. The effect of the Cys288—Leu and Cys288—Ile substitutions on bending of Phe267. The Phe267 side chain bends away from the wild-type position (——) to accommodate Leu (····) or Ile (-----) at position 288. Bending is more pronounced after Leu has been introduced because of Van der Waals overlaps involving the additional C32 of Leu.

Toma et al., 1991; Vriend et al., 1991) clearly show that models built on the basis of the crystal structure of thermolysin are sufficiently accurate for the design of site-directed mutations. Therefore, despite the uncertainties related to model-building, it seems appropriate to conclude from the present data that mutations aimed at improving the packing density of the hydrophobic core are not an effective way to stabilize Nprs. Combining the present with previously described results, it would appear that mutational strategies for stabilization of Nprs should focus on the surface of the protein.
References


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Note added in proof

Recently we found that the replacement of the bulky hydrophobic residues in the interior of the Npr-ste by alanine had only marginal effects on the thermostability, confirming the insensitivity of the Npr-ste towards mutations in its hydrophobic core.