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Cumulative stabilizing effects of hydrophobic interactions on the surface of the neutral protease from *Bacillus subtilis*

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Using genetically engineered mutants of the neutral protease from *Bacillus stearothermophilus* (BsteNP), it had been shown that the surface-exposed structural motif constituted by Phe63 embedded in a four amino acid hydrophobic pocket is critical for the thermal stability of the thermophilic neutral proteases from *Bacilli*. To measure the stabilizing contribution of each hydrophobic interaction taking place between Phe63 and the hydrophobic pocket, we grafted this structural motif in the neutral protease from the mesophile *Bacillus subtilis* (BsubNP). This was accomplished by first creating the Thr63→Phe mutant of BsubNP and then generating a series of mutants in which the four amino acids which in thermolysin surround Phe63 and form the hydrophobic pocket were added one after the other. By analysing the thermal stability of each mutant it was found that the 2°C destabilizing effect of the Thr63→Phe substitution was completely suppressed by the addition of the four amino acid hydrophobic pocket, each replacement providing a stabilizing contribution of approximately 0.8–1°C. These results are discussed in the light of the peculiar mechanism of thermal inactivation of proteolytic enzymes.

Keywords: *Bacillus subtilis*/hydrophobic interactions/neutral proteases/protein stability

Introduction

The thermal inactivation of proteolytic enzymes cannot be simply accounted for by general thermodynamic principles (Sanchez-Ruiz *et al.*, 1988). Rather, a more complex mechanism is proposed in which the local unfolding of the most flexible regions of the enzymes is followed by a rapid autoproteolytic attack at specific sites. These events generally trigger the complete degradation of the proteins. The results from limited proteolysis experiments are widely used as an indication of high flexibility and solvent accessibility sites on the protein structure (Fontana *et al.*, 1986; Van Berkel *et al.*, 1991; Abousalham *et al.*, 1992; Krook *et al.*, 1992; Mast *et al.*, 1992; Mottonen *et al.*, 1992; Corina *et al.*, 1993; Jaeger *et al.*, 1993; Zhao and Somerville, 1993; Schneider *et al.*, 1994). Moreover, modelling studies (Hubbard *et al.*, 1994) have suggested that large local motions proximate to the scissile bond are required for limited proteolysis.

The sites where autoproteolysis of neutral proteases takes place are generally located on surface flexible loops (Fontana *et al.*, 1986; Signor *et al.*, 1990; Van den Burg *et al.*, 1990). Therefore, in order to identify the most critical regions responsible for the thermal inactivation of proteases, a detailed analysis of surface loops appears to be important.

Various approaches can be followed to localize the sites most susceptible to autoproteolysis. Among these, the comparison of the amino acid sequences of homologous proteases from mesophilic and thermophilic organisms turns out to be particularly useful, especially when the three-dimensional structure of the enzymes under study is available.

In an attempt to highlight the structural elements which might contribute to the large difference in thermal stability between the neutral protease from *Bacillus subtilis* (BsubNP) and thermolysin (TLN), the neutral protease from *Bacillus thermoproteolyticus*, we have been scanning and comparing the surface regions of the two enzymes (Margarit *et al.*, 1992; Hardy *et al.*, 1994; F.Frigerio, I.Margarit, R.Nogarotto, G.Grandi, G.Vriend, G.Venema and V.G.H.Eijsink, in preparation). For example, in previous work (Toma *et al.*, 1991) a seven amino acid loop, which in BsubNP extends from residue 192 to residue 201 (see Figure 1) was replaced with the corresponding 10 amino acid, calcium-binding loop of TLN. The mutant produced was used to study the role of Ca^{2+} ion binding in the thermal stability of BsubNP.

In the present work, our attention was concentrated on the surface region of BsubNP surrounding and including Thr63 (throughout this work, the amino acid numbering of BsubNP and of the other neutral proteases refers to the numbering of TLN; for alignments see Figure 1). Van den Burg *et al.* (1991) demonstrated that this region is critical for the stability of thermophilic neutral proteases from *Bacilli*. In fact, they showed that when in BsteNP the solvent-exposed Thr63 was replaced with phenylalanine, the T_{50} of the mutated enzyme increased by 6.2°C. This remarkable stabilizing effect was attributed to the optimal hydrophobic contacts generated among the introduced aromatic ring and the side-chain methylene groups of the surrounding four amino acids Gln61, Val9, Gln17 and Arg11 (Van den Burg *et al.*, 1994). Interestingly, the X-ray structure of the highly stable TLN (Holmes and Matthews, 1982) clearly shows that in this enzyme the corresponding phenylalanine is nicely embedded in a hydrophobic pocket generated by the same residues present in BsteNP (Figure 2A).

In BsubNP, Thr63 and the surrounding residues create a highly polar environment which is completely different from what is observed in BsteNP and TLN (Figure 2B). This observation led us to predict that the replacement of Thr63 with Phe would have generated a destabilizing effect in BsubNP owing to the unfavourable contribution of an aromatic ring exposed to water (Reidhaar-Olson and Sauer, 1990). Such a destabilizing effect should be counteracted by the proper replacement of the four amino acids surrounding the aromatic ring with residues able to create a hydrophobic pocket similar to that present in TLN. If the four amino acids constituting the hydrophobic pocket are added one after the other and the

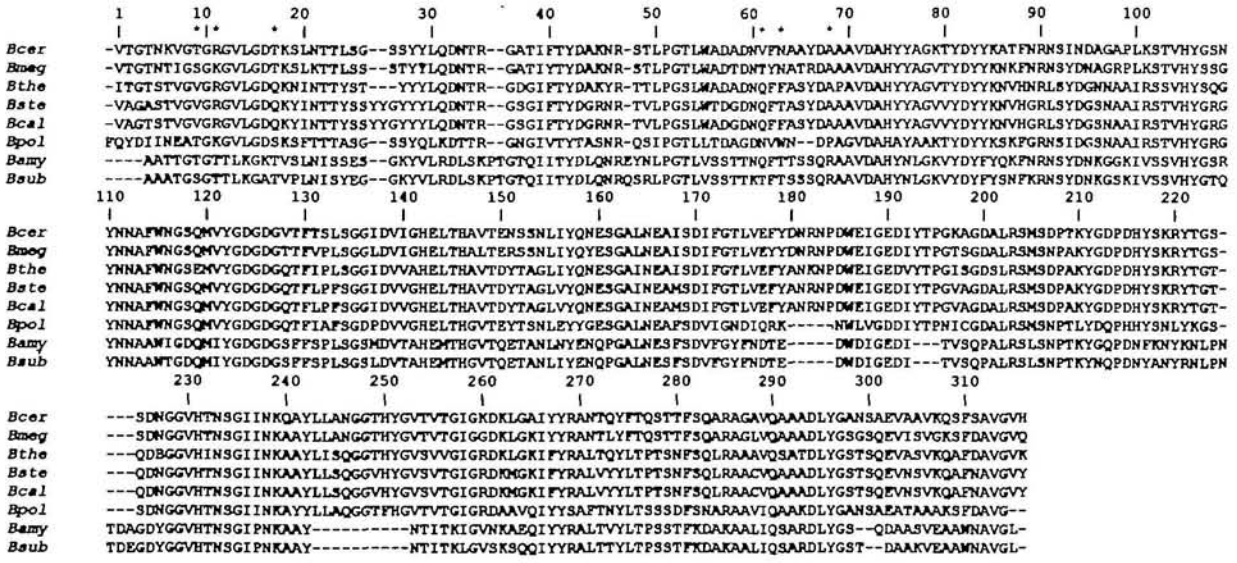

Fig. 1. Alignment of the amino acid sequences of the *Bacillus* zinc-dependent neutral proteases. Bcer, *B.cereus*; Bmeg, *B.megaterium*; Bthe, *B.thermoproteolyticus*; Bste, *B.stearothermophilus*; Bcal, *B.caldolyticus*; B.pol, *B.polymixa*; Bamy, *B.amyloliquefaciens*; Bsub, *B.subtilis*. Asterisks indicate the amino acids involved in the site-directed mutagenesis experiments described in this paper. For details, see text.

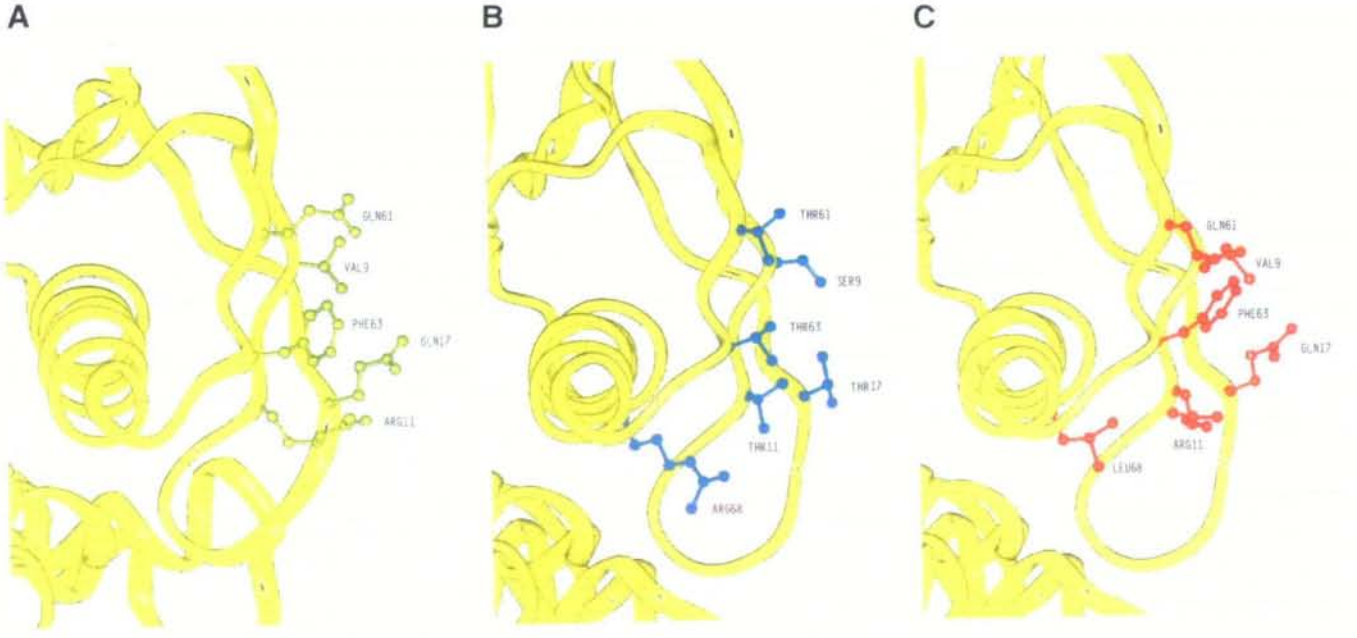

Fig. 2. Structural details of the region surrounding residue 63. (**A**) 3D structure of thermolysin; (**B**) model structure of wild-type BsubNP; (**C**): model structure of *mut6*.

thermal stability of the intermediate constructs is analysed, one can quantitate the contribution of the single hydrophobic interactions to the overall stability of the final mutant.

In accordance to the prediction analysis, here we show that the Phe63 mutant is 2.1°C less stable than the wild-type BsubNP. In addition, we also demonstrate that this mutant can be stabilized by a factor which is more or less proportional to the number of hydrophobic contacts generated by the specific substitutions around Phe63. As a final result, a net stabilization of approximately 1°C, compared with the wild-type BsubNP, is obtained.

Materials and methods

Plasmids and strains

The *B.subtilis* strain SMS108 (Npr-, His, Leu, recE4⁻) and plasmid pSM127 harbouring the neutral protease gene have been already described (Toma *et al.*, 1986).

Phage M13 mp8 and the *Escherichia coli* strain 71/18 (Messing, 1983) were used for site-directed mutagenesis. *B.subtilis* and *E.coli* were transformed following the procedures described by Contente and Dubnau (1979) and Hanahan (1983), respectively.

Site-directed mutagenesis

The methods used to obtain the BsubNP mutants have already been described (Toma *et al.*, 1989). The oligodeoxynucleotides used for site-directed mutagenesis were synthesized using the System One Plus synthesizer from Beckman. Most of them were designed either to create or to remove a restriction site so as to facilitate the selection of mutants. The sequences of the oligodeoxynucleotides used are the following:

Thr63→Phe:
5′ ACG.AAA.ACA.TTC.**TTC**.TCT.TCA.TCA.CAG.CGG 3′
Xmn1(Asp700) **Phe63**

Thr61→Gln:
5′ GTC.TCG.AGC.ACA.ACG.AAA.**CAA**.TTC.TTC.TCT.TCA 3′
HgiA1(AspH1) **Gln61**

Ser9→Val:
5′ CAT.GCG.GCC.GCC.ACT.GGA.**GTC**.GGA.ACA.ACT 3′
Not I **Val9**

Thr17→Gln
5′ CTA.AAG.GGC.GCA.CAG.GTA.CCT.TTG.AAC 3′
Gln17 *KpnI*

Thr11→Arg:
5′ GCC.ACT.GGA.GTC.GGC.**CGG**.ACT.CTA.AAG.GGC 3′
XmaIII **Arg11**

Arg68→Leu:
5′ TCA.TCA.CAG.**CTG**.GCA.GCC.GTC.GAC.GCA.CAC 3′
Leu68 *SalI*

Thr63→Phe oligonucleotide was used to create *mut1* starting from the wild-type neutral protease gene (plasmid pSM127) as template. For cumulative mutagenesis experiments the template for oligonucleotides Thr61→Gln, Ser9→Val, Thr17→Gln, Thr11→Arg and Arg68→Leu was the DNA from *mut1*, *mut2*, *mut3*, *mut4* and *mut5*, respectively. Finally, Arg68→Leu oligonucleotide was also used to create *mut7* from wild-type pSM127.

Enzyme production and purification

The *B.subtilis* strains harbouring either plasmid pSM127 or one of its derivatives were grown in 100 ml of VY medium (2.5% veal infusion broth, 0.5% yeast extract) supplemented with 5 μg/ml kanamycin and 5 mM $CaCl_2$, at 37°C for 16 h. The cells were removed by centrifugation and after addition of 1 mM PMSF the culture supernatants were extensively dialysed against 5 mM sodium acetate (NaOAc) (pH 5)–2.5 mM $CaCl_2$. The enzyme solutions were then loaded on to an S-Sepharose column (1×8 cm) equilibrated with 5 mM NaOAc (pH 5)– 2.5 mM $CaCl_2$ and the column was washed with three volumes of equilibration buffer. BsubNP and its mutants were finally eluted with 20 mM NaOAc (pH 5)–200 mM NaCl–5 mM $CaCl_2$. The protease-containing fractions were pooled and subsequently passed through a Sepharose–Gly–D-Phe (Walsh *et al.*, 1974) column (1×2 cm) equilibrated with 20 mM NaOAc (pH 5)–5 mM $CaCl_2$–10% propan-2-ol. The proteases were eluted with 20 mM NaOAc (pH 5)–5 mM $CaCl_2$–10% propan-2-ol–2.5 M NaCl.

All chromatographic steps were performed at 4°C at a flow-rate of 0.5 ml/min. The purified enzymes were analysed by SDS–PAGE as already described (Toma *et al.*, 1989) and stored at –80°C in the Gly–D-Phe elution buffer.

Enzyme activity and thermal stability

Proteolytic activity was determined using casein as substrate (Fujii *et al.*, 1983). The assay mixtures contained 1% casein in 25 mM Tris–HCl (pH 7.5)–2.5 mM $CaCl_2$ and protease samples at a final concentration of 2–10 μg/ml.

The thermal stability of the wild-type BsubNP and its mutants was determined by incubating the purified enzymes [2 – 10 μg/ml in 20 mM Tris–HCl (pH 7.5)–2.5 mM $CaCl_2$] for 30 min at various temperatures (from 50 to 65°C) and measuring the residual enzymatic activity with the casein assay. The residual activity is expressed as the percentage of the activity of the same solution kept at 37°C.

T_{50} is defined as the temperature at which 50% enzymatic activity is preserved after incubation for 30 min. The difference in thermal stability between two enzymes is expressed as ΔT_{50}, this being the mean value resulting from at least six independent determinations of the difference in the T_{50} values, as measured for both proteins in the same experiment. The estimated error is ±0.3°C.

Results

Design and construction of mutants

Figure 2A and B show the topological organization of TLN and BsubNP around residue 63, as determined by X-ray structural analysis and computer-assisted model building, respectively (Holmes and Matthews, 1982; F.Frigerio, I.Margarit, R.Nogarotto, G.Grandi, G.Vriend, G.Venema and V.G.H.Eijsink, in preparation). In TLN, Phe63 is accommodated in a hydrophobic pocket constituted by the aliphatic moieties of the side chains of Val9, Arg11, Gln17 and Gln61. The pocket provides optimal Van der Waals contacts with the aromatic ring of Phe63 and shields it from the solvent. In BsubNP, Thr63 is largely exposed to the solvent on a highly hydrophilic surface, where mostly threonine and serine side chains are present. Indeed, the accessible surface area for Thr63 side chain hydrophobic atoms, calculated on the BsubNP model with the ACCESS routine of program WHATIF (Vriend, 1986) is 61% of the area calculated for the unfolded conformation (14.0 and 23.1 $Å^2$, respectively).

On the basis of these structural features, the Thr63→Phe substitution (Table I, *mut1*), which in BsteNP is known to be stabilizing (Van den Burg *et al.*, 1994), is likely to have the opposite effect in BsubNP, owing to the unfavourable contribution given by a lipophilic group exposed to water (see Discussion). However, this destabilizing effect is expected to be counteracted if additional amino acid replacements are made which create a hydrophobic pocket around Phe63 (Figure 2C).

To test this hypothesis, a set of BsubNP mutants was produced (*mut2–mut6*) in which, according to the strategy described in Materials and methods, a series of substitutions was progressively added to the Thr63→Phe mutation. The mutants (see Table I) were created with the following assumptions:

mut2 A slight favourable stabilizing effect is expected for the partial shielding of the Phe63 aromatic ring by the amidic end of Gln61.

mut3 The methyl groups of Val9 should provide stabilizing hydrophobic contacts with Phe63 and Thr17.

mut4 Additional stabilizing hydrophobic contacts are expected to take place between Gln17 and the three side chains of Val9, Phe63 and Thr11.

Table I. Effect of amino acid substitutions on the thermal stability of *BsubNP*

Mutant	Amino acid substitutions	ΔT_{50}[a] versus wild-type (±0.3°C)
mut1	Thr63→Phe	−2.1
mut2	Thr63→Phe, Thr61→Gln	−2
mut3	Thr63→Phe, Thr61→Gln, Ser9→Val	−0.6
mut4	Thr63→Phe, Thr61→Gln, Ser9→Val, Thr17→Gln	+0.4
mut5	Thr63→Phe, Thr61→Gln, Ser9→Val, Thr17→Gln, Thr11→Arg	0
mut6	Thr63→Phe, Thr61→Gln, Ser9→Val, Thr17→Gln, Thr11→Arg, Arg68→Leu	+0.9
mut7	Arg68→Leu	−1

[a]Temperature at which 50% of the activity is lost after 30 min incubation.

mut5 The Thr11→Arg substitution is predicted to be almost neutral, owing to the opposite effects of the energetically favourable hydrophobic interaction between the arylic group of Phe63 and the alkylic group of Arg11, and the unfavourable charge repulsion between the guanidinium groups of Arg11 and Arg68.

mut6 The replacement of Arg68 with Leu is designed to stabilize the protein by removing the charge repulsion introduced with *mut5*. The side chain in position 68 displays no direct contact with the environment of Phe 63, except for residue 11.

mut7 The Arg68→Leu mutation was also introduced into the wild-type enzyme to measure the effect of this substitution alone on protease thermal stability.

The plasmids carrying the BsubNP mutated genes were used to transform neutral protease minus *B.subtilis* cells and the transformant colonies were selected on casein-containing plates. One colony of each transformation was randomly selected and used for subsequent characterization of the enzymes.

Enzyme purification and analysis

The wild-type neutral protease and its mutants were purified from the supernatant of 100 ml cultures, using the two-step purification procedure described in Materials and methods. The procedure, which consists of cation-exchange chromatography followed by Gly-D-Phe affinity chromatography, turned out to be rapid and efficient and 2–3 mg of the wild-type enzyme and its mutants were routinely obtained with purities higher than 95% (Figure 3). After purification, a specific activity of 85 000 U/mg was determined for all the proteases (data not shown).

Typical thermal stability curves as determined by residual activity measurements are shown in Figure 4 and the ΔT_{50} values of BsubNP mutants with respect to the wild-type enzyme are given in Table I.

The most stabilizing replacement appeared to be Ser9→Val, whose effect on protein stabilization was approximately 1.4°C, whereas the Thr61→Gln substitution had almost no effect. The Thr17→Gln replacement, which enhanced the protease stability by 0.8°C, ranked in the middle. Interestingly, the Thr11→Arg substitution contributed favourably to the protein stabilization only when the positively charged guanidinium group of Arg68 was replaced with the hydrophobic side chain of Leu (*mut6*, +0.5°C with respect to *mut4*). This suggests, as predicted by the 3D model inspection, that the side chains of Arg11 and Arg68 come into contact and that the repulsive force between the two positive charges overwhelms what is

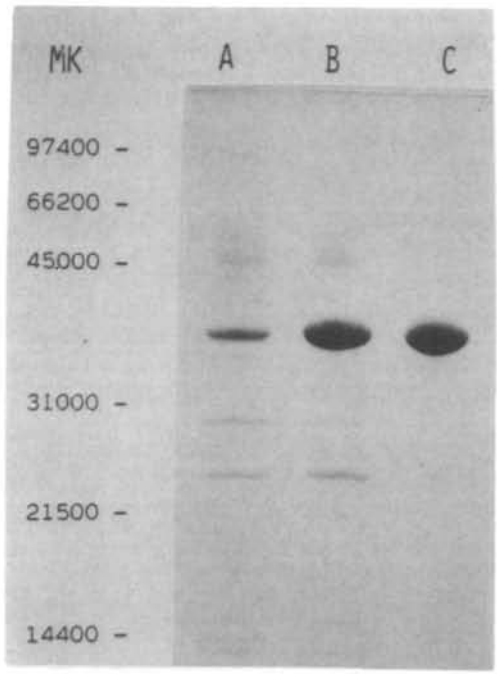

Fig. 3. SDS–PAGE analysis of the wild-type neutral protease after each purification step. 25 μg of proteins were loaded per lane. MK, molecular weight markers; A, dialysed supernatant; B, BsubNP sample after S-Sepharose F-F; C, BsubNP sample after Gly–D-Phe.

gained by the optimization of the hydrophobic contacts. It should be pointed out that since the Arg68→Leu substitution *per se* destabilized the protein by 1°C (*mut7*, Table I) probably because of its unfavourable effect on the free energy of solvation, the net contribution of the hydrophobic interactions generated by the Thr11→Arg mutation could be in the region of 1.5°C.

Similar results were obtained when fluorescence emission measurements (Margarit *et al.*, 1992) instead of residual activity experiments were used to follow the denaturation curves of the proteins (data not shown).

The relationship between thermal stability and buried hydrophobic surface area was investigated. For this purpose, the protein hydrophobic surface area buried upon folding was calculated (WHATIF: Vriend, 1986) for the models of wild-type BsubNP and *mut1–6* and plotted in Figure 5 together with the corresponding ΔT_{50} values. A linear correlation is clearly observable for *mut 1–6*.

Discussion

Although the role of the hydrophobic hydration of apolar groups on protein stability is still a matter of discussion (Dill,

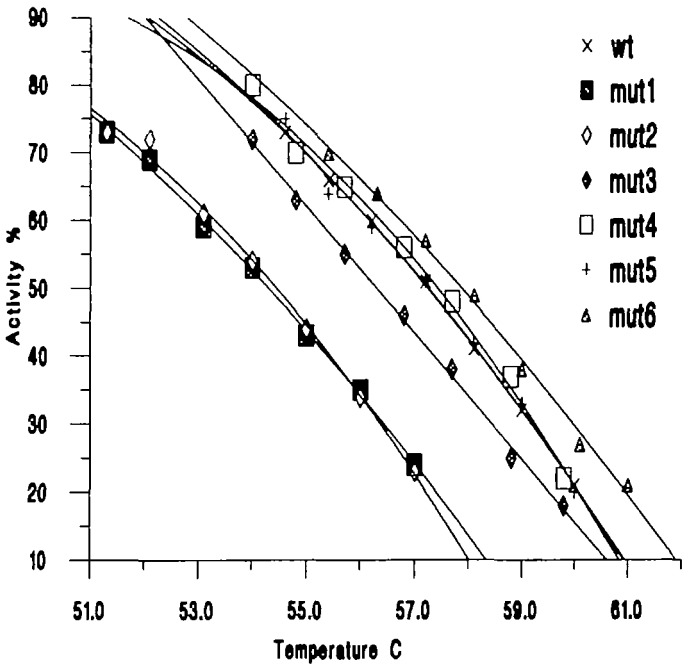

Fig. 4. Typical thermal stability curves of the wild-type BsubNP and its mutants. For details, see text.

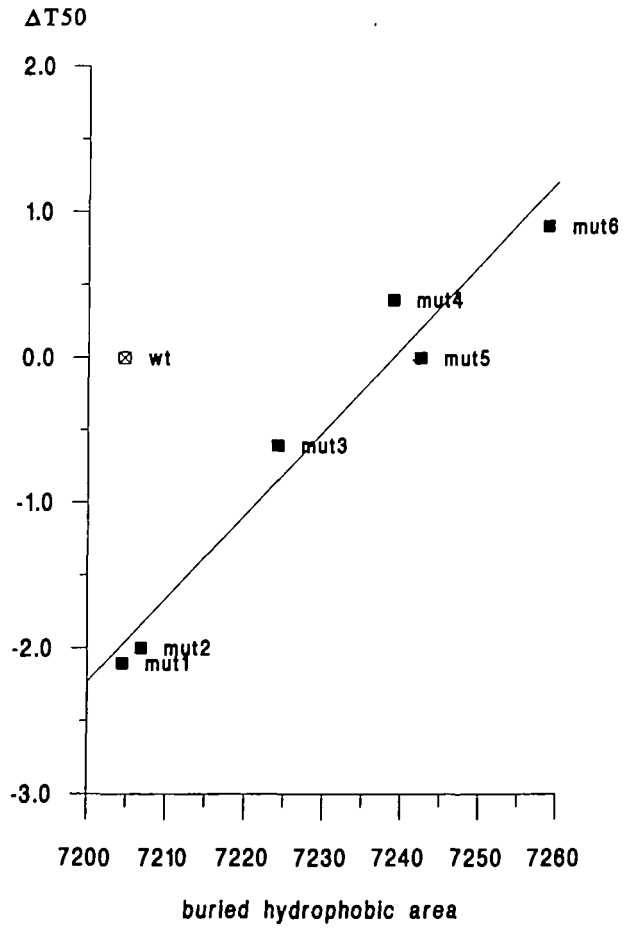

Fig. 5. Correlation between the change in protein hydrophobic surface area buried upon folding (Å^2) and the ΔT_{50} values (°C) of wild-type BsubNP and *mut1–6*. The correlation was calculated by linear regression for *mut1–6*.

1990; Creighton, 1991), it is generally believed that the presence of hydrophobic residues on the protein surface is unfavourable to the overall protein stability. In fact, by using protein engineering techniques it has been shown (Pakula and Sauer, 1990) that the destabilizing effect of amino acid replacements at the surface of a protein is proportional to the hydrophobicity index of the replacing amino acid, this index being defined as the amino acid transfer free energy from organic solvent to water (Fauchère and Pliska, 1983).

However, apparently in contradiction with this general assumption are the data by Van den Burg *et al.* (1994) showing that in BsteNP the stabilizing effect of amino acid substitutions of the solvent-exposed Thr63 is proportional to the degree of hydrophobicity of the replacing amino acid, the Phe substitution being the most stabilizing. These results, discussed by the authors on the basis of a BsteNP model structure (Vriend and Eijsink, 1993) derived from the TLN X-ray coordinates (Holmes and Matthews, 1982), were explained by attributing a crucial role to the side chains of the amino acids surrounding residue 63. According to Van den Burg *et al.* and in agreement with the crystallographic data for TLN, these amino acids form a hydrophobic pocket able to optimize the Van der Waals contacts with the aromatic ring of residue 63 and able to shield both residue 63 and the backbone β-sheet structure underneath from the water molecules.

To test experimentally the relevant role in the overall protein stability of the structural motif constituted by an aromatic ring embedded in a hydrophobic pocket, we took into consideration the *B.subtilis* neutral protease. Our model structure of this enzyme predicts that in the region around Thr63 the backbone structure nicely superimposes on the corresponding region in TLN. However, the threonine residue finds itself in an environment totally different from the TLN Phe63, creating together with the surrounding amino acids a highly polar surface.

Therefore, BsubNP appeared to us an excellent model to study and quantitate the contribution to protein stability of the hydrophobic interactions which can form around an aromatic ring.

This kind of analysis also offered us the opportunity to test whether or not the TLN structural motif of Phe63 surrounded by a hydrophobic pocket could have the same dramatic effect on protein stabilization as was the case for BsteNP.

As predicted, a gradual increase in thermal stablility was observed with the sequential introduction of hydrophobic interactions in *mut1–6*. Indeed, our results (Figure 5) indicate a linear correlation between an increase in buried hydrophobic surface and an increase in thermal stabilty, starting from the mutant protein Phe63.

The decreased stability of *mut1* with respect to wild type does not correspond to a lower buried hydrophobic surface area. An explanation can be suggested, which takes into account the possible destabilizing interactions between the solvent and the main-chain H-bond pattern in this region of BsubNP. In the case of wild type, the Thr63 side chain could mask the β-pleated sheet from water molecules with directional H-bonds, whereas in the case of *mut1*, Phe63 is not able to hinder the potentially denaturing interactions.

From an analysis of experimental protein structures, dense side chain clusters were detected (Heringa and Argos, 1991). Most of them were found near the protein surface and a correlation was shown between residue composition and side-chain volume and surface. The involvement of such clusters

in the stabilization of nearby secondary structure elements was suggested. The amino acids that were replaced around Phe63 in the series *mut2–7* may well build a surface-exposed cluster that helps in masking the β-sheet structure from the solvent.

The stability of the final mutant in which the hydrophobic pocket was fully reconstituted (*mut6*) was more than 3°C higher than that of the Thr63→Phe mutant and 1°C higher than that of the wild-type enzyme.

This result raises the question of why in BsubNP the TLN structural motif formed by Phe63 and its surrounding amino acids does not produce the same dramatic stabilizing effect found in BsteNP. One possible answer is that the positive contribution to protein stability given by each amino acid replacement is counteracted by some concomitant negative effects such as repulsive forces among side chains, backbone distorsion or reduced protein solvation. Indeed, the experiments described here suggest that this appears to be the case at least for the Arg68→Leu mutation.

However, an alternative explanation can be envisioned which takes into account the peculiar mechanism of thermal inactivation of proteases. Such a mechanism assumes that during the thermal denaturation process of proteases the partial unfolding of highly flexible local structures is the rate-determining step (Braxton and Wells, 1992; Eijsink *et al.*, 1992c) and that this step is immediately followed by fast proteolytic events.

In the case of the neutral proteases of mesophilic origin (BsubNp), the number of such labile structures is expected to be relatively high, compared with the homologous more stable enzymes (TLN, BsteNP). Moreover, the activation free energies associated with the unfolding of these structures are likely to have similar magnitudes. According to this mechanism, the favourable contribution of an amino acid replacement to local stability is not expected to affect the properties of the other labile regions as well as their local unfolding velocities and therefore it should not influence substantially the overall protein stability.

Thus, the extent of thermodynamic stabilization of specific amino acid substitutions cannot be fully appreciated because of the kinetic factors governing local unfolding and guiding to autoproteolysis. This would explain why, in general, the amino acid replacements on proteases of mesophilic origin produce limited increases in the T_{50} value of the mutated proteins (Toma *et al.* 1991; Eijsink *et al.*, 1992a,b; Margarit *et al.*, 1992; Siezen *et al.*, 1993; Hardy *et al.*, 1994; F.Frigerio, I.Margarit, R.Nogarotto, G.Grandi, G.Vriend, G.Venema and V.G.H.Eijsink, in preparation). The same postulated mechanism based on irreversible denaturation explains why, in contrast, more effective results could be obtained by stabilizing replacements on proteases of thermophilic origin such as BsteNP (Van den Burg *et al.*, 1994; Eijsink *et al.*, 1995).

A direct consequence of this latter explanation is that since the effects of even largely favourable interactions could be masked by the rapid kinetics of unfolding of the numerous labile sites located on the surface of the molecules, the proteases of mesophilic origin should not be recommended as model systems to study quantitatively the contribution of intramolecular interactions to protein stabilization.

However, an interesting aspect emerging from this work is that this is not necessarily always the case. In fact, the creation of destabilizing substitutions followed by the rational design of amino acid replacements addressed to stabilize the perturbed region might be utilized as a general approach to study quantitatively the contribution to thermal stability of specific amino acid interactions using proteases of mesophilic origin. In other words, by designing a destabilizing substitution one opens a window within which favourable amino acid interactions can be quantitatively studied, the amplitude of the window being given by the number of degrees Celsius lost by the wild-type enzyme as a result of the destabilizing mutation.

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References

Abousalham,A., Chaillan,C., Kerfelec,B., Foglizzo,E. and Chapus,C. (1992) *Protein Engng*, **5**, 105–111.
Braxton,S. and Wells,J.A. (1992) *Biochemistry*, **31**, 7796–7801.
Contente,S. and Dubnau,D. (1979) *Mol. Gen. Genet.*, **167**, 251–258.
Corina,K., Grossman,S.R., Barsoum,J., Prakash,S.S., Androphy,E.J. and Pepinsky,R.B. (1993) *Virology*, **197**, 391–396.
Creighton,T.E. (1991) , *Curr. Opin. Struct. Biol.*, **1**, 5–16.
Dill,K.A. (1990) *Biochemistry*, **29**, 7133–7155.
Eijsink,V.G.H., Vriend,G., Van den Burg,B., Van der Zee,J.R., Veltman,O R., Stulp,B.K. and Venema,G. (1992a) *Protein Engng*, **5**, 157–163.
Eijsink,V.G.H., Vriend,G., Van den Burg,B., Van der Zee,J.R., and Venema,G. (1992b) *Protein Engng*, **5**, 165–170.
Eijsink,V.G.H., Vriend,G., Van der Vinne,B., Hazes,B., Van den Burg,B. and Venema,G. (1992c) *Proteins: Struct. Funct. Genet.*, **14**, 224–236.
Eijsink,V.G.H., Veltman,O.R., Aukema,W., Vriend,G. and Venema,G. (1995) *Nature Struct. Biol.*, in press.
Fauchère,J.L. and Pliska,V.E. (1983) *Eur. J. Med. Chem.*, **18**, 369–375.
Fontana,A., Fassina,G., Vita,C., Dalzoppo,D., Zamai,M. and Zambonin,M. (1986) *Biochemistry*, **25**, 1847–1851.
Fujı,M., Takagı,M., Imanaka,T. and Aiba,S. (1983) *J. Bacteriol.*, **154**, 831–837.
Hanahan,D. (1983) *J. Mol. Biol.*, **166**, 557–580.
Heringa,J. and Argos,P. (1991) *J. Mol. Biol.*, **220**, 151–171.
Hardy,F., Vriend,G., Van der Vinne,B., Frigerio,F., Grandı,G., Venema,G. and Eijsink,V.G.H. (1994) *Protein Engng*, **7**, 425–430.
Holmes,M.A. and Matthews,B.W. (1982) *J Mol. Biol.*, **160**, 623–639.
Hubbard,S.J., Eisenmenger,F. and Thornton,J.M. (1994) *Protein Sci.*, **3**, 757–768.
Jaeger,K.E., Ransac,S., Koch,H.B., Ferrato,F. and Dijkstra,B.W. (1993) *FEBS Lett.*, **332**, 143–149.
Krook,M., Prozorovski,V., Atrian,S., Gonzalez-Duarte,R. and Joernvall,H. (1992) *Eur. J. Biochem.*, **209**, 233–239.
Margarit,I., Campagnoli,S., Frigerio,F., Grandi,G., De Filippis,V. and Fontana,A. (1992) *Protein Engng*, **5**, 543–550.
Mast,A.E., Enghild,J.J. and Salvesen,G. (1992) *Biochemistry*, **31**, 2720–2728.
Messing,J. (1983) *Methods Enzymol.*, **101**, 20–78.
Mottonen,J., Strand,A., Symersky,J., Sweet,R.M., Danley,D.E., Geoghegan,K.F., Gerard,R.D. and Goldsmith,E.J. (1992) *Nature*, **355**, 270–273.
Pakula,A.A. and Sauer,R.T. (1990) *Nature*, **344**, 363–364.
Reidhaar-Olson,J.F. and Sauer,R.T. (1990) *Proteins*, **7**, 306–316.
Sanchez-Ruiz,J.M., Lopez-Lacomba,J.L., Cortijo,M. and Mateo,P.L. (1988) *Biochemistry*, **27**, 1648–1652.
Schneider,E., Wilken,S. and Schmid,R. (1994) *J. Biol. Chem.*, **269**, 20456–20461.
Siezen,R.J., Bruinenberg,P.G., Vos,P., Van Alen-Boerrigter,I., Nijhuis,M., Alting,A.C., Exterkate,F.A. and De Vos,W.M. (1993) *Protein Engng*, **6**, 927–937.
Signor, G., Vita,C., Fontana,A., Frigerio,F., Bolognesi,M., Toma,S., Gianna,R., De Gregoriis,R. and Grandi,G. (1990) *Eur. J. Biochem.*, **189**, 221–227.
Toma,S., Del Bue,M., Pirola,A. and Grandi,G. (1986) *J. Bacteriol.*, **167**, 740–743.
Toma,S., Campagnoli,S., De Gregoriis,E., Gianna,R., Margarit,I., Zamai,M. and Grandi,G. (1989) *Protein Engng*, **2**, 359–364.
Toma,S., Campagnoli,S., Margarit,I., Gianna,R., Grandi,G., Bolognesi,M., De Filippis,V. and Fontana,A. (1991) *Biochemistry*, **30**, 97–106.
Van Berkel,W.J.H., Regelink,A.G., Beintema,J.J. and de Kok,A. (1991) *Eur. J. Biochem.*, **202**, 1049–1055.
Van den Burg,B., Eijsink,V.G.H., Stulp,B.K. and Venema,G. (1990) *Biochem. J.*, **272**, 93–97.

Van den Burg,B., Enequist,H.G., Van der Haar,M.E., Eijsink,V.G.H., Stulp,B.K. and Venema,G. (1991) *J. Bacteriol.*, **173**, 4107–4115.
Van den Burg,B., Dijkstra,B.W., Vriend,G., Van der Vinne,B., Venema,G. and Eijsink,V.G.H. (1994) *Eur. J. Biochem.*, **220**, 981–985.
Vriend,G. (1990) *J. Mol. Graphics*, **8**, 52–56.
Vriend,G. and Eijsink,V.G.H. (1993) *J. Comput.-Aided Mol. Des.*, **7**, 367–396.
Walsh,K.A., Burstein,Y. and Pangburn,M.K. (1974) *Methods Enzymol.*, **34**, 435–440.
Zhao,G.P. and Somerville,R.L. (1993) *J. Biol. Chem.*, **268**, 14912–14920.

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