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Protein stabilization by hydrophobic interactions at the surface

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The contribution of the solvent-exposed residue 63 to thermal stability of the thermolysin-like neutral protease of *Bacillus stearothermophilus* was studied by analyzing the effect of twelve different amino acid substitutions at this position. The thermal stability of the enzyme was increased considerably by introducing Arg, Lys or bulky hydrophobic amino acids. In general, the effects of the mutations showed that hydrophobic contacts in this surface-located region of the protein are a major determinant of thermal stability. This observation contrasts with general concepts concerning the contribution of surface-located residues and surface hydrophobicity to protein stability and indicates new ways for protein stabilization by site-directed mutagenesis.

Bacillus neutral proteases (NP) are metalloendopeptidases that resemble thermolysin, the well-characterized extremely thermostable NP of *Bacillus stearothermophilus*. The crystal structures of thermolysin (Matthews et al., 1972; Holmes and Matthews, 1982) and of the NP from *Bacillus cereus* (Pauptit et al., 1988; Stark et al., 1992) have been elucidated, and on the basis of these structures three-dimensional models of other NP have been built (Signor et al., 1990; Vriend and Eijsink, 1993). Within the family of NP large differences in thermal stability occur and therefore these enzymes form an interesting system to study structure-stability relationships. Recent protein-engineering studies have revealed some of the structural factors that determine the thermal stability of NP (Imanaka et al., 1986; Toma et al., 1991; Van den Burg et al., 1991; Eijsink et al., 1992; Hardy et al., 1993; Vriend and Eijsink, 1993). In one of these studies Van den Burg et al. (1991) showed that a Thr→Phe substitution at the surface-located position 63 in the NP of *B. stearothermophilus* (NP-*ste*) had a large effect on thermal stability.

Amino acid substitutions at the surface usually do not affect the stability of proteins by large amounts (Perutz and Lehmann, 1968; Hecht et al., 1983; Alber et al., 1987; Bowie et al., 1990; Reidhaar-Olson and Sauer, 1990; Rennell et al., 1991). Nevertheless, it is generally believed that the introduction of hydrophobic residues at the surface of a protein is unfavourable for protein stability (Bashford et al., 1987; Reidhaar-Olson and Sauer, 1990; Pakula and Sauer, 1990; Creighton, 1990). The effect of the Thr→Phe substitu-

tion in NP-*ste* contrasts with both these general assumptions: thermal stability was increased after replacing a moderately hydrophilic threonine at the surface by a hydrophobic phenylalanine, and this increase was quite substantial (Van den Burg et al., 1991). In the present study the mechanism underlying the remarkable stabilizing effect of the Thr63→Phe substitution was investigated. For this purpose, eleven additional NP-*ste* variants differing at position 63 were constructed and characterized, and possible structural effects of the mutations were analyzed.

At elevated temperatures, NP are irreversibly inactivated as a result of autolysis, which complicates thermodynamic analysis of NP thermal stability (Dahlquist et al., 1976; Van den Burg et al., 1990; Eijsink et al., 1991). It has been shown that the rate of thermal inactivation is determined by the rate of local unfolding processes that render the NP molecule susceptible to autolysis (Vriend and Eijsink, 1993). In the present study, thermal stability of NP is characterized by t_{50} , the temperature at which 50% of an initial amount of proteolytic activity is preserved during a 30-min incubation. The t_{50} of thermolysin (which has Phe at position 63) was found to be 82°C, whereas for NP-*ste* (85% sequence identity with thermolysin) a t_{50} of 68.5°C was determined (Eijsink et al., 1993). The Thr63→Phe substitution was shown to contribute 6.2°C to the difference in thermal stability between the two enzymes (Van den Burg et al., 1991).

MATERIALS AND METHODS

Genetics

The NP-*ste npr* gene was originally cloned and sequenced by Fujii et al. (1983) and Takagi et al. (1985), respectively. All methods for subcloning, site-directed mutagenesis and expression of the *npr* gene have been described elsewhere (Eijsink et al., 1992). For site-directed mutagenesis both specific oligonucleotides and oligonucleotides with a degenerate codon for residue 63 were used.

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Abbreviations. NP, neutral protease; NP-*ste*, neutral protease of *B. stearothermophilus*; BHSA, buried hydrophobic surface area.

Enzymes. Neutral protease of *B. stearothermophilus* (3.4.24.28); thermolysin, neutral protease of *B. thermoproteolyticus* (EC 3.4.24.27).

Production and characterization of neutral proteases

Production, purification and subsequent characterization of the NP were performed as described earlier (Van den Burg et al., 1989; Eijnsink et al., 1992). For the determination of thermal stability, samples of diluted purified enzyme [approximately 0.1 μ M enzyme in 20 mM sodium acetate, pH 5.0, 5 mM CaCl₂, 0.5% (by vol.) isopropanol, 62.5 mM NaCl] were incubated for 30 min at various temperatures. Subsequently the residual protease activity was determined using a casein assay (Fujii et al., 1983). The t_{50} used to quantify thermal stability of the NP corresponds to the temperature of incubation at which 50% of the initial protease activity was preserved. The thermal stability of mutant NP was expressed as Δt_{50} , being the difference in t_{50} between the wild-type and the mutant enzyme. The error margin in the Δt_{50} values is approximately 0.3 °C. Attempts to measure reversible unfolding of NP failed because of the unavoidable occurrence of autolysis under denaturing conditions (see for example Dahlquist et al., 1976).

SDS/PAGE was performed as described previously (Van den Burg et al., 1989).

Protein concentrations were determined with the Micro BCA protein assay reagent (Pierce), using bovine serum albumin as a standard.

Structure analysis and molecular modelling

All modelling procedures were performed using WHAT IF (Vriend, 1990), essentially as described by Vriend and Eijnsink (1993). For modelling of NP-*ste* variants the crystal structure of thermolysin (Phe63; 85% overall sequence identity; Holmes and Matthews, 1982) was used as a starting point. Residues at position 63 were modelled using position-specific rotamer searches. The side chains were placed in the most preferred rotamer attainable without the introduction of unfavourable van der Waals' overlaps. The models were refined by a 500-step conjugate-gradient energy minimization using the GROMOS software (Van Gunsteren and Berendsen, 1987).

Solvent-accessible surface areas were determined using the surface-accessibility module of WHAT IF, which gives the same results as the Conolly surface-area-calculation package (Conolly, 1983). Buried surface areas were calculated by subtracting accessible surface areas from vacuum accessibility values of Gly-Xaa-Gly tripeptides, where Xaa is the residue evaluated (Chothia, 1974). Subsequently, the hydrophobic buried surface area (carbon and sulphur atoms) was integrated over residue 63 and the residues in its direct vicinity.

Throughout this study, residues are numbered according to the thermolysin sequence (Titani et al., 1972). Compared to thermolysin, NP-*ste* contains three extra residues, inserted in the 25–30 region (Takagi et al., 1985).

RESULTS

Characterization of mutant enzymes

All mutant NP were similar to the wild-type enzyme with respect to their electrophoretic mobility during SDS/PAGE and their specific activity to casein. Table 1 shows that the thermal stability of NP-*ste* was drastically affected by the changes at position 63, with the mutational effects on t_{50} ranging from -10°C to $+7.1^{\circ}\text{C}$ for the Pro and Arg mutants,

Table 1. Changes in thermal stability associated with mutations at position 63 in the neutral protease of *B. stearothermophilus*. Δt_{50} values given are the average of at least three independent assays. The error in Δt_{50} values was estimated to be 0.3 °C.

Residue at position 63	Δt_{50} °C
Arg	+ 7.1
Lys	+ 6.7
Phe ^a	+ 6.2
Ile	+ 4.1
Tyr	+ 3.6
Met	+ 1.5
Thr (wild-type)	0
Val	- 0.9
Glu	- 1.4
Ser	- 4.0
Ala	- 7.5
Asp	- 7.5
Pro ^b	-10.0

^a See also Van den Burg et al. (1991)

^b See also Hardy et al. (1993).

respectively. In general, amino acids with large hydrophobic side chains or with side chains containing long aliphatic moieties (arginine, lysine) appeared to be favourable for thermal stability. Apparently, the presence of methyl groups at γ and δ positions contributes strongly to thermal stability, as can be seen by comparing the thermal stabilities of the Ser mutant with wild-type enzyme, the Ala with the Val mutant, and the Val with the Ile mutant.

Structural considerations

Structural aspects of the mutations at position 63 were studied using the refined crystal structure of thermolysin (Matthews et al., 1972; Holmes and Matthews, 1982). This NP has Phe63, and all residues in the direct vicinity of Phe63 are the same as in NP-*ste*. Therefore, the highly similar NP-*ste* and thermolysin are expected to have the same three-dimensional structure in the environment of residue 63. As shown in Fig. 1, Phe63 is positioned at the surface of the protein, its side chain covering an underlying β -pleated structure. The side chain is partly buried because of the presence of several protruding surface residues which have their methyl groups in contact with the aromatic ring of the phenylalanine (Table 2). Val9 and the hydrophobic moieties of the residues Arg11, Gln17 and Gln61 form a hydrophobic pocket at the surface of the protein in which the aromatic ring of Phe63 resides. The polar groups of the arginine and the two glutamines confer hydrophilicity to the surface, permitting favourable interactions with the solvent.

Modelling studies (see Materials and Methods) showed that all residues listed in Table 1, except proline, can be accommodated at position 63 in thermolysin, without the introduction of steric clashes and without the need to position any of the residues involved (nos 9, 11, 17, 61, 63) in an unfavourable rotamer. For all mutants the amount of hydrophobic surface area that is buried upon folding was calculated. There appeared to be a clear correlation between the increase in buried hydrophobic surface area and thermal stability for most residues (Fig. 2).

The large destabilizing effect of the Thr63→Pro substitution is probably due to the fact that the main chain ϕ torsion

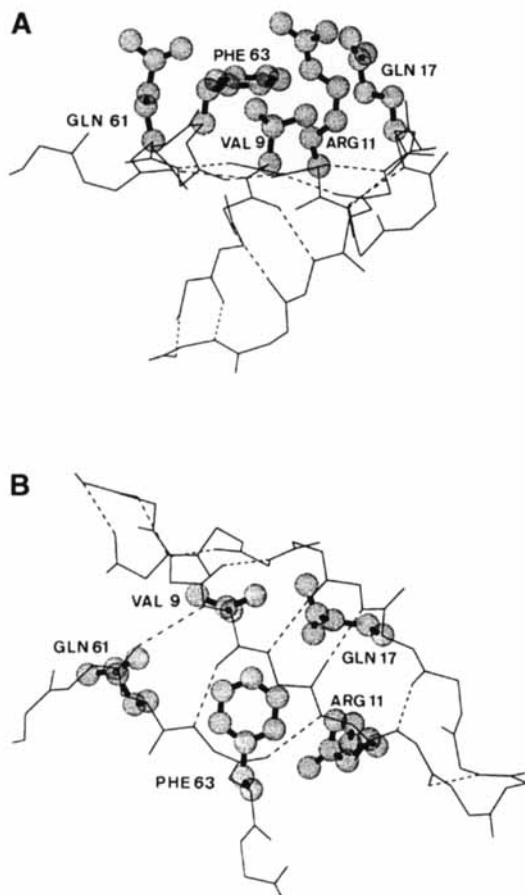


Fig. 1. Two views on Phe63 and surrounding residues in thermolysin. In (A) the view is along the surface of the protein, in (B) the view is from the solvent onto the protein. Dotted lines indicate hydrogen bonds. Hydrophobic side-chain–side-chain contacts involving Phe63 are listed in Table 2. The environments of residue 63 are completely identical in thermolysin and NP-*ste* (85% overall sequence identity), including all the residues of which the side chain is shown in this figure.

Table 2. Distances between carbon atoms in the side chain of Phe63 and carbon atoms in the surrounding side chains. Distances (0.45-nm cutoff) were taken from the crystal structure of thermolysin. All surrounding residues mentioned in this table are identical in thermolysin and NP-*ste* and they are located in regions where the two enzymes exhibit a high degree of sequence identity. Therefore, it is to be expected that thermolysin and the Phe63 mutant of NP-*ste* are almost identical with respect to the local structure around residue 63 (see also Vriend and Eijnsink, 1993).

Atoms of Phe63	Atoms of surrounding residue	Respective distances
		nm
C γ	Arg11-C ζ	0.45
C δ 1	Arg11-C β , C γ , C δ , C ζ	0.37, 0.45, 0.42, 0.35
C δ 2	Gln61-C β	0.42
C ϵ 1	Arg11-C β , C δ , C ζ	0.42, 0.44, 0.39
	Gln17-C γ , C δ	0.41, 0.42
C ϵ 2	Val9-C β	0.43
	Gln61-C β	0.43
C ζ	Val9-C β , C γ 1	0.41, 0.40
	Gln17-C γ , C δ	0.43, 0.42

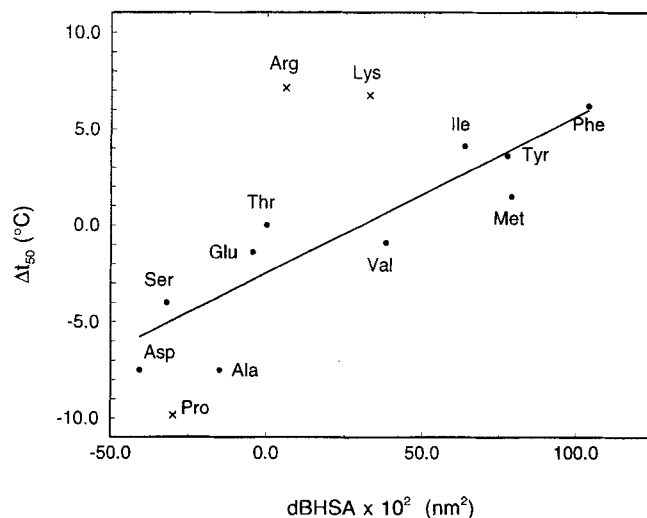


Fig. 2. Correlation between the change in thermal stability (Δt_{50}) and the change in buried hydrophobic surface area gained upon folding (dBHSA) in the neutral protease of *B. stearrowthermophilus* (NP-*ste*). The wild-type, having a threonine at position 63, was taken as a reference ($\Delta t_{50} = 0$; dBHSA = 0). The correlation was determined by a linear regression through the points indicated (●). Crosses indicate residues that are discussed separately in the text.

angle for residue 63 in the wild-type enzyme ($\phi = -109^\circ$) deviates rather strongly from the ϕ angle of about -65° preferred by the conformationally highly restricted proline (Hardy et al., 1993). This mutation is therefore expected to introduce conformational strain that will offset positive effects resulting from the hydrophobic contributions.

The Arg and Lys mutants are more stable than would be expected on the basis of the correlation shown in Fig. 2. Modelling studies of these mutants showed several favourable hydrophobic interactions between the aliphatic moieties of arginine and lysine and the methyl groups of the surrounding residues. However, these interactions seemed to be weaker and to involve less surface than in for example the Phe mutant. The models of the mutant enzymes indicated that the polar end groups of arginine and lysine are solvent exposed and that they can participate in hydrogen bonds with the side-chain oxygens of Gln17 and Gln61.

DISCUSSION

The correlation between buried hydrophobic surface area and thermal stability shown in Fig. 2 indicates the importance of hydrophobic contacts involving the surface-located residue 63. The tightly packed hydrophobic layer involving the carbon atoms in the side chain of residue 63 separates the hydrophilic protein surface and the solvent from the rest of the protein. It is conceivable that the correlation between thermal stability and buried hydrophobic surface area shown in Fig. 2 reflects the extent to which the β -sheet structures underneath the hydrophobic layer (Fig. 1) are shielded from invading water molecules and unfolding. Alternatively, it could be envisaged that the hydrophobic contacts involving residue 63 exert a stabilizing effect by reducing unfolding processes that involve the short surface loop (residues 63–69) that follows residue 63.

It is unlikely that hydrogen-bond formation with the solvent-exposed side-chain oxygens of Gln17 and Gln61 accounts for the high thermal stability of the Arg and Lys mu-

tants, because the effects of solvent-exposed hydrogen bonds are small. The fact that at position 63 positively charged residues (Arg, Lys) stabilize, whereas negatively charged residues (Glu, Asp) destabilize, suggests that electrostatic interactions play a role. Modelling studies (using position-specific rotamer searches for all residues involved; Vriend and Eijsink, 1993) showed no obvious short-range electrostatic interactions involving residue 63, but revealed that in principle, interactions with Arg11 and Asp59 could occur. Electrostatic interactions with Arg11 would be unfavourable in the Arg and Lys mutants and can obviously not explain why these mutants are so stable. Positioned in a different rotamer than the one used in our model of the Arg mutant, arginine at position 63 could form a reasonable salt bridge with Asp59. A similar salt bridge could not easily be envisaged in the Lys mutant. Since the Arg and Lys mutants are almost equally stable, it seems that salt-bridge formation with Asp59, if it occurs at all, is of minor importance. It should be noted that the polar groups of residues 11, 59 and 63 are all solvent exposed and that it is now generally accepted that electrostatic interactions at the surface of a protein are of minor importance for stability (Matthews, 1993).

The previous considerations indicate that hydrogen-bond formation and electrostatic interactions cannot explain why the Arg and Lys mutants are so stable. We propose that the favourable solvation of the polar end groups is an important cause for the high stability of the Arg and Lys mutants. This favourable solvation contrasts with the unfavourable solvation of some hydrophobic groups in the mutants containing hydrophobic residues at position 63 and may therefore explain why the Arg and Lys mutants are so unexpectedly stable. Favourable solvation could in principle also occur in the labile Asp and Glu mutants. However, the side chains of glutamic acid and especially aspartic acid are considerably shorter than those of arginine and lysine. Therefore, their polar end groups have unfavourable interactions with the hydrophobic moieties of the residues surrounding position 63, and they are not well solvated. Modelling of the Glu mutant showed unfavourable close contacts between the carboxyl group of the glutamic acid and Val9. The high stability of the Arg and Lys mutants indicates the importance of the hydrophobicity of the side chains of arginine and lysine and the dual character of these residues.

Considering the broad specificity of NP, conformational features rather than sequence characteristics of the NP molecule determine the sites of auto-proteolytic attack (Fontana, 1988). In accordance with this, it has been shown that local unfolding processes, that render the molecule susceptible to autolysis, are the rate-limiting step in the thermal-inactivation process (Dahlquist et al., 1976; Eijsink et al., 1992; Braxton and Wells, 1992; Vriend and Eijsink, 1993). Thus, the magnitude of t_{50} is determined by the activation free energy of local unfolding processes which result in conformations susceptible to autolysis. Changes in t_{50} are therefore directly related to changes in the resistance of the NP molecule to thermally induced unfolding.

As the unfolding processes that determine the rate of thermal inactivation have a local character, the effect of a mutation on t_{50} is dependent on the location of the mutation. Mutations that affect a relatively weak region of the enzyme (or, more precisely, that affect a local unfolding pathway with a relatively low activation energy; Eijsink et al., 1992) have relatively large effects on t_{50} . The surface-located residue 63 is part of a weak region of NP-*ste* (Hardy et al., 1993; Vriend and Eijsink, 1993) and it should therefore be noted that the

hypothetical free-energy differences for global unfolding that are associated with the mutations described above may be smaller than suggested by the drastic effects on t_{50} . In principle, mutations could also affect thermal stability by a direct effect on the cleavability of the mutated chain segment. Considering the (lack of) sequence specificity of NP (Heinrikson, 1977) this is highly unlikely in the present case.

Identifying the structural elements that contribute to the extreme thermal stability of thermolysin has been a major challenge ever since the unravelling of the three-dimensional structure of this extremely stable *Bacillus* NP (e.g. Matthews et al., 1974). The data recently obtained in our laboratories, which are presented here and elsewhere (Van den Burg et al., 1991; Hardy et al., 1993) clearly show that the difference in thermal stability between NP-*ste* and thermolysin is not determined by general factors such as the number of intramolecular hydrogen bonds or salt-bridges. Instead, just a few surface-located structure elements, such as the hydrophobic cluster around residue 63, seem to be crucial for thermal stability.

Protein-engineering studies have provided considerable information about the contribution of specific interactions and structural elements to protein stability (e.g. Matthews, 1993). A structural element similar to the one described here has not yet been analyzed by site-directed mutagenesis experiments. Extensive studies of mutant proteins, including an analysis of all available structures of mutant T4 lysozymes (e.g. Alber, 1989; Matthews, 1993; De Filippis, Sander and Vriend, unpublished results), have not revealed to us mutations that are comparable with the ones in the present study. Recently, Blaber et al. (1993) replaced a highly solvent-exposed residue in an α -helix in T4 lysozyme by all 19 alternative amino acids and concluded that the side-chain hydrophobic surface buried against the side of the helix contributes considerably to stability. The present study provides evidence that the conclusion of Blaber et al. (1993) also applies to other surface residues that may not be helical or fully solvent exposed but that do have surface-located hydrophobic contacts.

The present data have uncovered a new way of enhancing protein stability by showing that hydrophobic clustering at the surface, involving hydrophobic residues and the hydrophobic moieties of hydrophilic residues, can be a major stabilizing factor. They show that surface and near-surface hydrophobicity are worth consideration in the design of strategies for protein stabilization. The present data highlight the specific dual character of arginine and lysine and they form an illustration of the increasingly accepted concept that every site-directed mutation for the stabilization of a protein should be evaluated in its own specific structural context and not on the basis of certain general rules.

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