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Cumulative stabilizing effects of glycine to alanine substitutions in *Bacillus subtilis* neutral protease

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Oligonucleotide-directed mutagenesis has been used to replace glycine residues by alanine in neutral protease from *Bacillus subtilis*. One Gly to Ala substitution (G147A) was located in a helical region of the protein, while the other (G189A) was in a loop. The effects of mutational substitutions on the functional, conformational and stability properties of the enzyme have been investigated using enzymatic assays and spectroscopic measurements. Single substitutions of both Gly147 and Gly189 with Ala residues affect the enzyme kinetic properties using synthetic peptides as substrates. When Gly replacements were concurrently introduced at both positions, the kinetic characteristics of the double mutant were roughly intermediate between those of the two single mutants, and similar to those of the wild-type protease. Both mutants G147A and G189A were found to be more stable towards irreversible thermal inactivation/unfolding than the wild-type species. Moreover, the stabilizing effect of the Gly to Ala substitution was roughly additive in the double mutant G147A/G189A, which shows a 3.2°C increase in T_m with respect to the wild-type protein. These findings indicate that the Gly to Ala substitution can be used as a strategy to stabilize globular proteins. The possible mechanisms of protein stabilization are also discussed.

Key words: neutral protease/protein engineering/protein stability/proteolytic enzymes/site-directed mutagenesis

Introduction

The advent of genetic methods to exchange at will amino acid residues of proteins has allowed numerous site-directed mutagenesis experiments aimed at dissecting the role and magnitude of the forces and interactions which determine the protein-specific structure and stability (Matthews, 1987, 1991; Alber, 1989; Pakula and Sauer, 1989; Fontana, 1991a, b). The outcome of these studies should be rewarding from both the fundamental and practical points of view. In fact, an understanding of the protein stability problem is a prerequisite to unravelling the mechanisms of protein folding (Creighton, 1990; Jaenicke, 1991a, b) and designing *de novo* protein molecules with specific structure and function (De Grado, 1988). Moreover, protein stability is of considerable practical interest because protein lability hampers successful applications of enzymes in various fields of chemical technology, the food industry, medicine, analytical chemistry, etc. (Nosoh and Sekiguchi, 1990).

Protein stability can also be investigated using the structure–stability relationships in naturally occurring stable proteins, produced in nature by thermophilic and in general extremophilic bacteria. Indeed, comparative analyses of the molecular structures and properties of thermophilic enzymes (and proteins) with those of their counterparts from mesophilic sources allowed the inference of the role of key interactions contributing to protein stability and the highlighting of some strategies followed by nature in engineering protein stability by random mutation and natural selection of protein molecules (Menéndez-Arias and Argos, 1989; Fontana, 1991a, b; Jaenicke, 1991a, b).

The results of both the protein engineering studies and the analyses of structure–stability properties of thermophilic enzymes and proteins, have provided some useful guidelines and methods for the study of protein stability (Fontana, 1991a, b). These include the introduction of stabilizing disulfide bonds into the protein at strategic positions, the improvement of hydrophobic packing or hydrogen bonding, the stabilization of helix dipoles with appropriately charged residues positioned at helix termini, the substitution of specific residues in the protein molecule to allow the formation of specific salt bridges and the introduction into a protein of metal ion binding sites (Fontana, 1991a, b; Jaenicke, 1991a, b). One strategy that has already proved successful in engineering protein stability involves the replacement of glycine residues with amino acids containing a β-carbon, mostly alanine (Hecht *et al.*, 1986; Imanaka *et al.*, 1986; Matthews *et al.*, 1987; Takagi and Imanaka, 1989; Ganter and Plückthun, 1990). The rationale for this approach is that this replacement causes a decrease in the chain entropy of the unfolded state, thus shifting the equilibrium to the folded state, because glycine lacks a β-carbon and has more backbone conformational flexibility. For this reason more free energy is required during the folding process to restrict the conformation of glycine rather than alanine (or other branched amino acids) in the folded protein (Matthews *et al.*, 1987). Alternatively, with Gly to Ala substitutions located at helices in globular proteins, the cause of protein stabilization would be the introduction of a good helix-former in place of a helix-breaker (Hecht *et al.*, 1986; Imanaka *et al.*, 1986).

The family of neutral proteases isolated from both mesophilic and thermophilic bacteria and showing a varied degree of stability appears to be a convenient system to use to study protein structure–stability properties and relationships. Indeed, detailed analyses of the functional and stability properties and the amino acid sequences are available for the proteases produced by *Bacillus thermoproteolyticus* (thermolysin) (Titani *et al.*, 1972), *B.stearothermophilus* (Takagi *et al.*, 1985), *B.caldolyticus* (Van den Burg *et al.*, 1991), *B.cereus* (Sidler *et al.*, 1986), *B.amyloliquefaciens* (Vasantha *et al.*, 1984), *B.mesentericus* (Stoeva *et al.*, 1990) and *B.subtilis* (Yang *et al.*, 1984). Moreover, high-resolution X-ray structures of thermolysin (Matthews *et al.*, 1972; Holmes and Matthews, 1982) and *B.cereus* protease (Pauptit *et al.*, 1988) have been reported. Finally, the genes of the protease from *B.subtilis* (Toma *et al.*, 1986) and *B.stearothermophilus* (Takagi *et al.*, 1985) have been cloned, which has made possible some successful protein engineering experiments (Imanaka *et al.*, 1986; Takagi and Imanaka, 1989; Toma *et al.*, 1989, 1991; Eijsink *et al.*, 1990, 1991).

In this paper the replacement of glycine residues with alanine in *B.subtilis* neutral protease is reported and functional and thermal stability data for single (G147A and G189A) and double (G147A/G189A) protein mutants presented. It is shown that the Gly to Ala substitutions located either in a helix (G147A) or in a loop (G189A) lead to enhanced heat stability of the protein, and moreover that multiple substitutions (G147A/G189A) have a cumulative effect. It is concluded from this and previous work that Gly to Ala replacements constitute a useful strategy to stabilize globular proteins. The mechanistic basis of the protein stabilization is discussed.

Materials and methods

Materials

The wild-type neutral protease (NP) used in this study was expressed by *B.subtilis* SMS108 containing plasmid pSM127 (Toma *et al.*, 1986), purified to homogeneity as previously described (Toma *et al.*, 1986, 1989; Signor *et al.*, 1990) and stored at −20°C.

Tris and Mops *o*-phenanthroline were obtained from Fluka (Basel, Switzerland) and the materials used for SDS−PAGE were acquired from Bio-Rad (Richmond, CA). The proteins used as molecular mass markers for SDS−PAGE were purchased from Pharmacia (Uppsala, Sweden). Solvents for reversed-phase HPLC were obtained from Merck (Darmstadt, Germany). The synthetic peptide substrates (Morgan and Fruton, 1978) Z−Phe−Leu−Ala−OH, Z−Ala−Leu−Ala−OH, Z−Gly−Leu−Ala−OH and Z−Val−Leu−Ala−OH were obtained from Bachem (Bubendorf, Switzerland). The affinity column Sepharose−Gly−D-Phe was prepared according to Walsh *et al.* (1974). All other chemicals and reagents were of analytical grade and obtained from C.Erba (Milan, Italy) or Merck.

Methods

SDS−PAGE was performed using the tricine system (Schägger and Von Jagow, 1987). Protein samples (~10 μg) of both wild-type and mutant NP were prepared in the sample buffer containing 5 mM *o*-phenanthroline to avoid autodigestion of the protease, heated at 100°C for 5 min and loaded onto a 16.5% SDS−PAGE gel (Signor *et al.*, 1990). After electrophoresis, the gels were stained with Coomassie brilliant blue R-250.

Reversed-phase HPLC was performed using an LKB system (Bromma, Sweden) and a reverse-phase Vydac C_{18} column (4.6 × 105 mm) (The Separation Group, Hesperia, CA). Solvent A was 0.05% aqueous trifluoroacetic acid (TFA) and solvent B was 0.05% TFA in acetonitrile. The protein was eluted by the application of a linear gradient of acetonitrile in 0.05% TFA from 20% (held for 5 min) to 50% (for 30 min) at a flow rate of 1 ml/min.

Circular dichroism spectra in the far-UV region were obtained at room temperature on a Jasco J-500A recording spectropolarimeter equipped with a data processor Model DP-501 as previously described (Toma *et al.*, 1991). A quartz cell of 0.1 cm path length was used and the protein concentration was 0.1 mg/ml.

Fluorescence emission measurements were performed on a Perkin−Elmer Model MPF-66 spectrofluorimeter equipped with a cell holder containing a thermostat. Spectra were recorded using excitation wavelengths of 280 and 295 nm and the band width for both excitation and emission was 10 nm. Spectra were corrected by subtracting the spectrum of buffer only (Fontana *et al.*, 1977).

Preparation of mutants of neutral protease

Site-directed mutagenesis was performed using the method of Zoller and Smith (1983) with the following oligonucleotides to direct mutations at Gly residues in positions 147 and 189 of the wild-type NP chain: G147A, 5′-CATGAAATGAC*GCATGCA*-GTCACCCAAGAAACA-3′; G189A, 5′-GAAGACTGG*GATA-TATCGCA*GAAGACATTACGGTC-3′. The double mutant G147A/G189A was prepared using single-stranded DNA containing the Gly to Ala mutation in position 189 as the template and the oligonucleotide used to achieve the Gly147 to Ala mutation as the primer. The oligonucleotides were synthesized using a Beckman One Plus synthesizer. Mutants were identified by sequencing their ssDNA according to the chain termination method (Sanger and Coulson, 1975), utilizing the 5′-GGTTCAT-TCTTCTCTCCG-3′ chain, which hybridizes next to the Gly147 codon, as the oligonucleotide primer.

The mutated *Hin*dIII−*Bam*HI fragment of the NP gene was introduced back into pSM127, producing plasmids pSM374 (G147A), pSM375(G189A) and pSM376(G147A/G189A). These plasmids were again analyzed by sequencing and used to transform the NP minus *B.subtilis* SMS108 strain, which was grown for production of mutant NP essentially following the procedure described by Toma *et al.* (1986, 1989, 1991) and Signor *et al.* (1990). In a typical experiment, ammonium sulfate was added (80% saturation) to the supernatant of a 2 l culture obtained by 24 h incubation at 37°C and the precipitate recovered by centrifugation. The precipitate was resuspended and extensively dialyzed against 50 mM Tris−HCl buffer, pH 7.5, containing 5 mM calcium acetate. The protein material was then chromatographed on a DEAE−Sephadex A-25 (Pharmacia) column equilibrated and eluted with the same Tris buffer. Under these conditions, the wild-type and mutant NP were not retained in the column. The protease-containing fractions were subsequently passed through a Sephadex G-100 (Pharmacia) column and finally chromatographed on the affinity column Sepharose−Gly−D-Phe (Walsh *et al.*, 1974).

The amino acid sequence numbering used in this study is that of the 316-residue chain of thermolysin (Titani *et al.*, 1972), which shows ~50% sequence identity to the 300-residue chain of *B.subtilis* NP (Yang *et al.*, 1984). The mutated residues 147 and 189 (thermolysin numbering) correspond to residues 148 and 185 in the *B.subtilis* NP chain respectively.

Enzyme assays

The kinetic parameters, K_m and k_{cat}, of both wild-type and mutant NP were determined using the synthetic peptide substrates Z−Phe−Leu−Ala−OH, Z−Ala−Leu−Ala−OH, Z−Gly−Leu−Ala−OH and Z−Val−Leu−Ala−OH (Morgan and Fruton, 1978), and following the hydrolysis of the X−Leu peptide bond by HPLC analysis as previously described (Toma *et al.*, 1991). The activity was measured at 37°C in 50 mM Tris−HCl buffer, pH 7.0, 2.5 mM calcium acetate. The data were fitted to the Michaelis−Menten model to derive the kinetic constants. Enzyme concentrations were determined spectrophotometrically using the coefficient $A^{1\%}_{280\ nm}$ = 13.8 (Toma *et al.*, 1991) to permit calculation of k_{cat} from the relationship $k_{cat} = V_{max}/[enzyme]$. Alternatively, proteolytic activity was determined using casein as substrate (Toma *et al.*, 1986). The assay mixture contained 0.6% casein in 50 mM Tris−HCl buffer, pH 7.0, containing 2 mM calcium acetate. The protease (5−10 μg) was added to 5 ml of the assay solution and, after incubation at 37°C for 10 min, TCA (5 ml, 10% in water) was added to precipitate the undigested casein. After centrifugation,

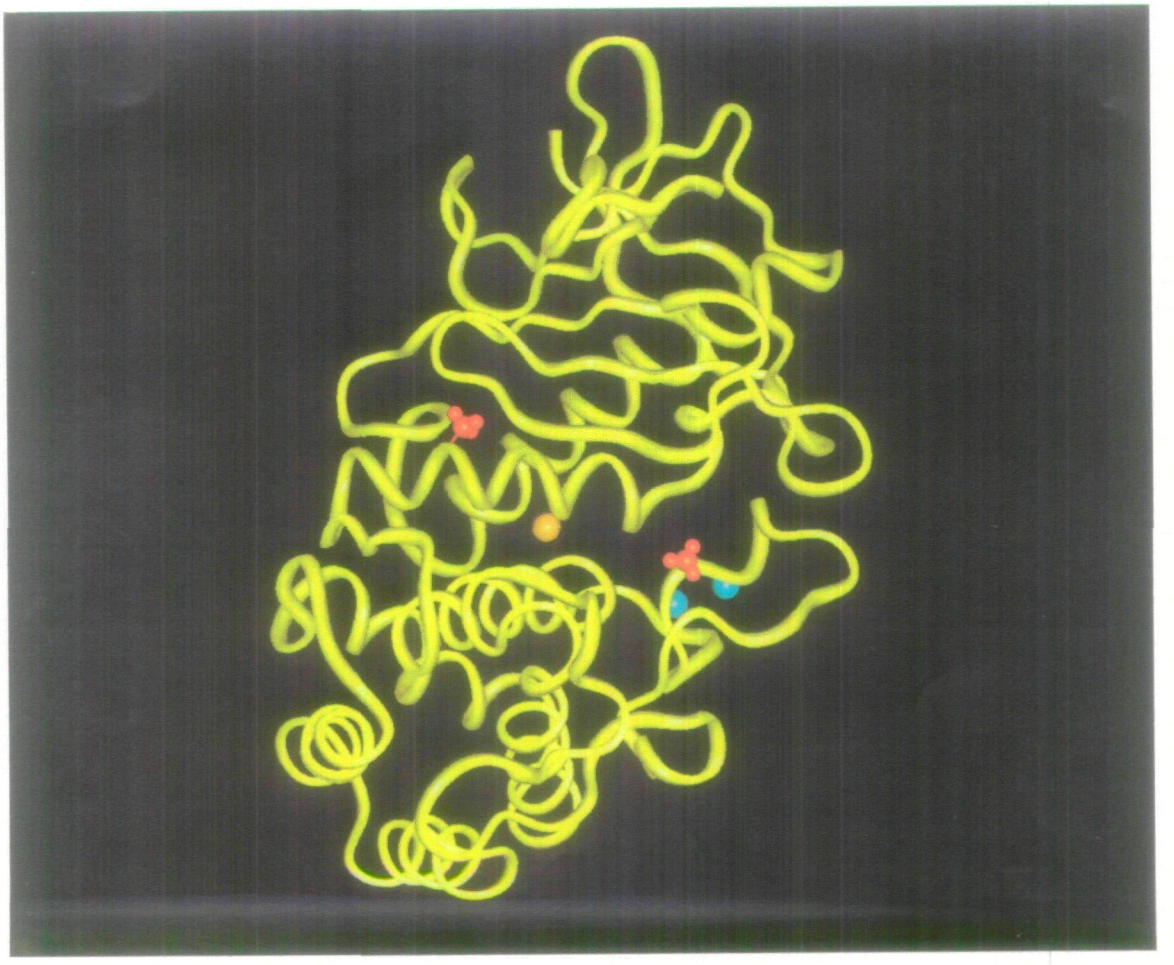

Fig. 1. Model structure of the *B.subtilis* NP molecule The model was obtained using a Silicon Graphics IRIS SD/35 workstation using the software program INSIGHT-II (Biosym, 1991). Yellow, α-carbon trace; red, Ala147 and Ala189 side chains; gold, Zn ion in the active site cleft; cyan, two calcium ions.

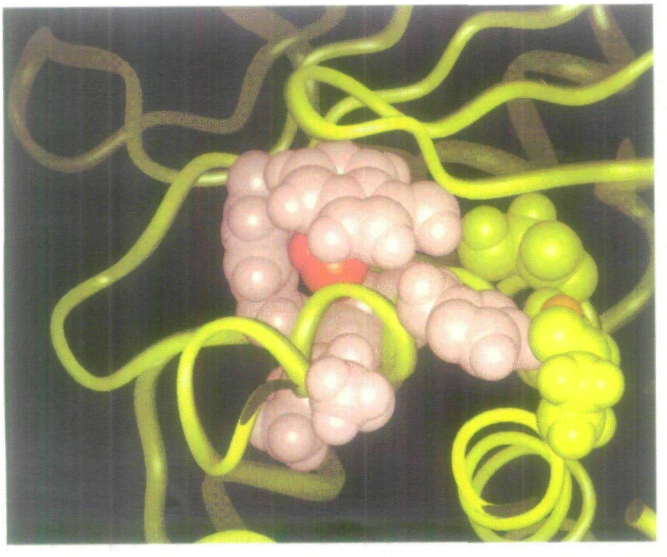

Fig. 7. Details of the environment at the site of mutation in G147A. Yellow, side chains of His142 and Glu166 coordinating the Zn ion (gold) and of the catalytic Glu143, pink, side chains of residues contacting Ala147, Val148, Thr149, His146, Tyr 93, Met145 and (above the methyl group of Ala147, shown in red) Trp115

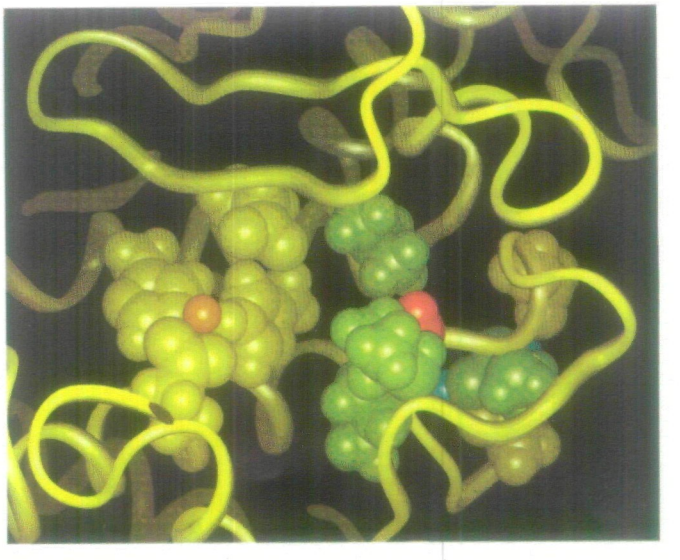

Fig. 8. Details of the environment of the site of mutation in G189A. Yellow, His142, His146, Glu166, Glu143 and the two calcium ions (cyan spheres) coordinating residues Asp191, Asp138 and Asp185; green, the side chains of residues in van der Waals contact with Ala189 (shown in red), Val139, Leu202, Ile188 and Glu186.

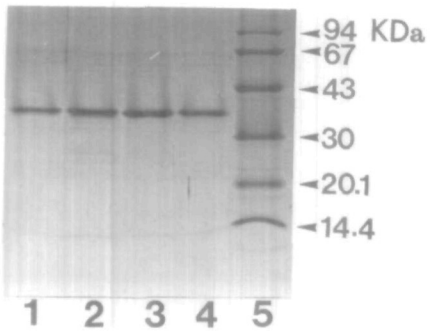

Fig. 2. SDS−PAGE analysis of wild-type and mutant *B.subtilis* NP. Protein samples (~10 μg) purified by affinity chromatography were loaded on 16.5% acrylamide gels using the tricine system and the gels stained with Coomassie brilliant blue. (1) Wild-type; (2) G189A; (3) G147A; (4) G147A/G189A; (5) protein molecular mass markers (indicated in kDa, left).

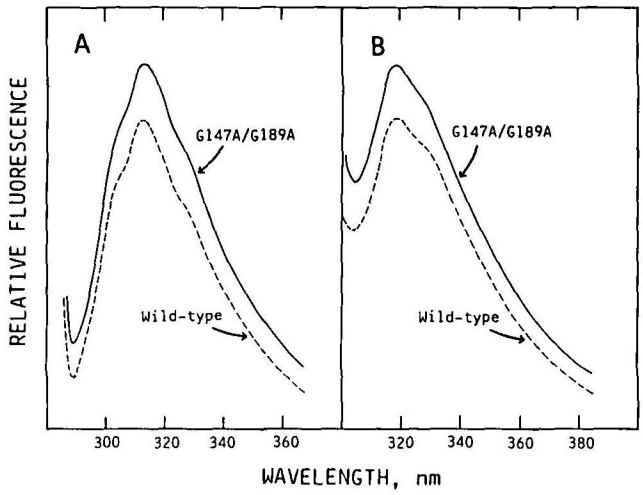

Fig. 3. Fluorescence emission spectra of wild-type and G147A/G189A mutant *B.subtilis* NP. The protein was dissolved (4 μg/ml) in 10 mM Mops buffer, pH 7.5, containing 25 mM NaCl and 10 mM $CaCl_2$. Fluorescence spectra were recorded at 20°C with an excitation wavelength of 280 (A) and 295 nm (B) respectively. ---, wild-type; ——, G147A/G189A. Tracings of the spectra are slightly offset for clarity, as indicated

the amount of digested protein was determined in the supernatant by spectrophotometric reading at 275 nm. One unit of proteolytic activity was defined as the amount of enzyme yielding an increase of 0.001 in the absorbance at 275 nm per min at 37°C.

Thermal stability measurements

The heat inactivation of both wild-type and mutant NP was monitored by activity measurements. The enzyme was dissolved (1 mg/ml) in 25 mM Tris−HCl buffer, pH 7.5, containing 2.5 mM calcium acetate, and incubated at 61°C in closed vials to avoid evaporation. At intervals aliquots were removed from the solution, chilled on ice and assayed at 37°C for residual activity using casein as substrate.

The melting profile of NP samples was determined by measuring the temperature dependence of the intensity of fluorescence emission at 320 nm upon excitation at 295 nm. The protein was dissolved (10 μg/ml) in 10 mM Mops buffer, pH 7.0, containing 10 mM of both NaCl and $CaCl_2$, and placed in the cuvette of the spectrofluorimeter. The temperature of the solution inside the cuvette was continuously increased at a constant rate of 2°C/min using a circulating water bath, and monitored by a thermistor probe inserted directly into the cell, with an accuracy of ±0.1°C.

The kinetics of the thermal denaturation/unfolding at 58°C was determined with protein samples dissolved (4 μg/ml) in 10 mM Mops buffer, pH 7.5, containing 10 mM of both NaCl and $CaCl_2$.

Results

Preparation and isolation of mutants of B.subtilis and neutral protease

Neutral protease from *B.subtilis* is a single polypeptide chain of 300 residues and contains 25 Gly residues (Yang *et al.*, 1984). Two positions on the chain were chosen for the Gly to Ala replacement: positions 147 and 189, located in a helical segment and a loop respectively (Figure 1). The structural characteristics of these mutation sites were derived from a 3-D model (Biosym, 1991) of the NP, constructed on the basis of the known structure of the related thermolysin molecule (Matthews *et al.*, 1972; Holmes and Matthews, 1982), which shows ~50% sequence identity with *B.subtilis* NP (Yang *et al.*, 1984; Signor *et al.*, 1990). The two replacements chosen allow the investigation of the effects of the same substitution in regions of the protein with different conformations (see Discussion).

Genes encoding single (G147A, G189A) or double (G147A/G189A) mutations of NP were prepared by oligonucleotide-directed mutagenesis following standard procedures (see Materials and methods). The plasmid DNA for each mutant was sequenced in its entirety to check the desired mutations at the gene level. The three mutants were expressed in yields comparable to those of the wild-type (Toma *et al.*, 1986) and were purified to homogeneity by ion exchange, gel filtration and affinity chromatography on Sepharose−Gly−D-Phe following procedures already described for both wild-type (Toma *et al.*, 1986) and mutant NP (Toma *et al.*, 1989, 1991). When purified protein samples of both wild-type and mutant NP were analyzed by reversed-phase HPLC on a Vydac C_{18} column (see Materials and methods), single symmetrical peaks of protein material were eluted from the column (not shown). Mutant and wild-type NP show identical mobility on SDS−PAGE (Figure 2).

Conformational studies

The overall conformational properties of the three mutants of NP were evaluated using CD and fluorescence emission measurements. In the far-UV region (200−250 nm) the mutants show CD spectra identical in shape and ellipticity values to that of the wild-type species (not shown). The CD spectra of all protein species show the characteristics of helical polypeptides, with minima near 208 and 220 nm (Greenfield and Fasman, 1969) and $[\theta]_{220\ nm}$ = 14 000 ± 300° cm^2/dmol.

The fluorescence emission spectra of both wild-type and double mutant G147A/G189A are shown in Figure 3. *Bacillus subtilis* NP contains three tryptophan residues located at positions 115, 186 and 310 (thermolysin sequence numbering). Upon excitation at 280 nm, both tyrosine (22 residues) and tryptophan residues contribute to the fluorescence emission spectrum (Figure 3A), whereas excitation at 295 nm gives a fluorescence spectrum resulting from the contribution of the indole fluorophores only (Figure 3B) (Burstein *et al.*, 1973; Brand and Witholt, 1974; Fontana *et al.*, 1977). The fluorescence spectra shown in Figure 3, for both double mutant and wild-type NP, are essentially identical. Analogous identity of spectra is observed for the single mutants G147A and G189A (not shown for clarity). The maxima of emission, observed at 318 and 320 nm upon excitation at 280 and 295 nm respectively, are quite unusual for

Table I. Kinetic properties of wild-type and mutant *B.subtilis* neutral protease[a]

Substrate Z−P1−P1′−P2′	Wild-type			G147A			G189A			G147A/G189A		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Z−Phe−Leu−Ala−OH	1.4	1986	1418	9.4	1933	205.6	2 5	2139	845	0.95	1038	1084
Z−Ala−Leu−Ala−OH	11.4	3005	263	202	3500	17.3	1 5	2694	1761	8.7	1329	152.4
Z−Gly−Leu−Ala−OH	21.3	1150	54	35.3	105 8	3.0	2.4	1847	776	4 4	186.4	42.5
Z−Val−Leu−Ala−OH	2.8	6.1	2 18	24	3.76	0.16	0.96	13.1	13.6	7.0	7.6	1.1

[a]The kinetic parameters were determined using synthetic peptide substrates at 37°C in Tris buffer, pH 7.0, as described in Materials and methods. The initial substrate concentrations used were 0.2−20 mM and enzyme concentrations were 0.04−5 μg/ml.

globular proteins and, in particular, indicate that tryptophan residues are located in the interior of the protein in a rather hydrophobic environment. Moreover, with spectra recorded at an excitation wavelength of 295 nm (Figure 3B), the major peak at 320 nm is accompanied by a shoulder near 330 nm, indicating heterogeneity of both emission properties and microenvironment of tryptophan residues of the protein (Burstein *et al.*, 1973; Brand and Witholt, 1974). Thus, because the fluorescence emission spectra were essentially identical in both intensity and maximum wavelength of emission for both wild-type and mutant NP, it can be concluded that the 3-D structures of these protein species are similar.

Enzymatic properties

The effect of Gly to Ala substitutions on the enzymatic properties of mutant NP were evaluated using synthetic peptides as substrates (Morihara and Tsuzuki, 1970; Morgan and Fruton, 1978). The results shown in Table I indicate that Gly replacements at both positions 147 and 189 affect K_m and k_{cat} parameters. This is a consequence of the fact that the nearby residues are involved either in the active site or in the hydrophobic binding site of the enzyme (Colman *et al.*, 1972) (see also Discussion). The data show that mutant G147A has a reduced catalytic efficiency towards the synthetic peptide substrates because of a 10-fold increase in K_m value, with the exception of Z−Gly−Leu−Ala−OH where k_{cat} is mostly affected. On the contrary, mutant G189A shows a significant enhancement in the affinity for substrates having a small amino acid (Gly, Ala) in position P1. In the double mutant G147A/G189A the catalytic efficiency of the wild-type enzyme is somewhat restored.

The enzymatic activity of both wild-type and mutant NP was also determined using casein as substrate (see Materials and methods). The specific activities of wild-type and mutants G147A, G189A and G147A/G189A were 80 000 ± 4000, 54 000 ± 2700, 97 000 ± 5000 and 110 000 ± 5500 U/mg protein respectively.

Thermal stability

To compare the effects of the Gly substitutions with thermal stability, mutant and wild-type NP were incubated at 61°C under otherwise identical conditions and the remaining enzymatic activity measured (Figure 4). All three mutants show clearly enhanced heat stability with respect to that of the wild-type enzyme, the double mutant G147A/G189A being the most stable enzyme. Moreover, the ranking of the half-life ($t_{1/2}$) at 61°C is G147A/G189A > G147A > G189A > wild-type, with values of $t_{1/2}$ clearly showing a cumulative effect on thermostability (Table II). Similar results were obtained by incubating the enzymes at 55 or 58°C (data not shown).

The enhancement of protein stability caused by the Gly replacement was also evidenced by analyzing the melting profile (Figure 5) and the kinetics of denaturation/unfolding (Figure 6)

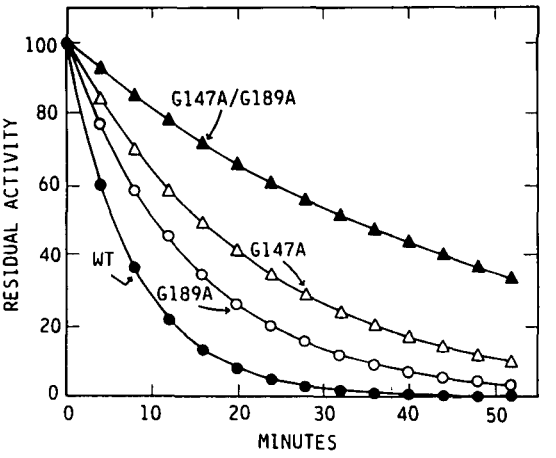

Fig. 4. Kinetics of the thermal inactivation of wild-type and mutant *B.subtilis* NP. The protein was dissolved (1 mg/ml) in 25 mM Tris−HCl buffer, pH 7.5, containing 2.5 mM calcium acetate and heated at 61°C in a closed vial. At intervals, aliquots (50 μl) were removed from the reaction mixture and assayed at 37°C for residual enzymatic activity using casein as substrate (see Materials and methods). Residual activities are expressed as percent of the initial activity ●, wild-type; ○, G189A; △, G147A, ▲, G147A/G189A.

Table II. Stability of wild-type and mutant *B.subtilis* neutral protease[a]

Protein	T_m (°C)	ΔT_m (°C)	$t_{1/2}$ (min)
Wild-type	66 0	–	5.2
G189A	67.1	1.1	10.5
G147A	68.2	2.2	15.2
G147A/G189A	69.2	3 2	33.0

[a]Melting temperatures (T_m) were evaluated from protein thermal denaturation profiles as given by fluorescence measurements (see legend to Figure 5 and Materials and methods for experimental details). The accuracy of T_m figures is ± 0.1°C. ΔT_m values represent the changes in T_m of mutant protease with respect to the wild-type species. The half-life ($t_{1/2}$) of protein samples heated at 61°C was determined by assaying residual activity. The protein was dissolved (1 mg/ml) in 25 mM Tris−HCl buffer, pH 7.5, containing 2.5 mM calcium acetate, and incubated at 61°C in a closed vial At intervals, aliquots were taken from the solution and assayed for activity using casein as substrate (see also legend to Figure 4 and Materials and methods)

of the various enzyme species by fluorescence emission measurements. Figure 5 shows the irreversible melting profile of both the wild-type and the double mutant G147A/G189A determined by monitoring the fluorescence emission intensity on increasing the temperature. Similar melting curves were obtained for the single mutants G147A and G189A (not shown). It can be observed that, in the temperature range 25−55°C, there is a monotonous decay of fluorescence intensity caused by thermal

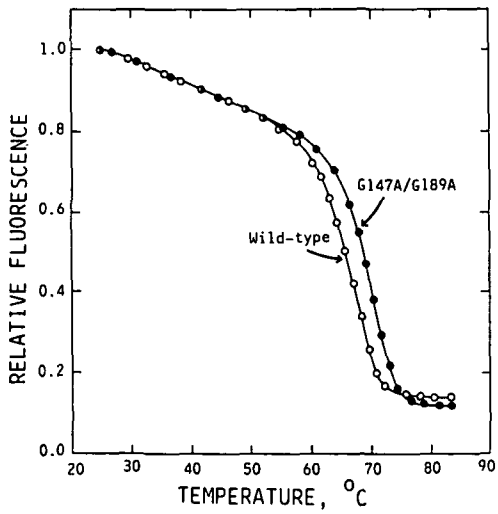

Fig. 5. Melting profile of wild-type and G147A/G189A mutant *B.subtilis* NP. The thermal denaturation/unfolding was monitored by measuring the intrinsic fluorescence emission intensity at 320 nm upon excitation at 295 nm of protein samples dissolved (10 μg/ml) in 10 mM Mops buffer, pH 7.0, containing 10 mM of both NaCl and $CaCl_2$. The protein sample was heated in the cuvette at a constant rate of 2°C/min and the fluorescence intensity was measured continuously. ○, wild-type; ●, G147A/G189A.

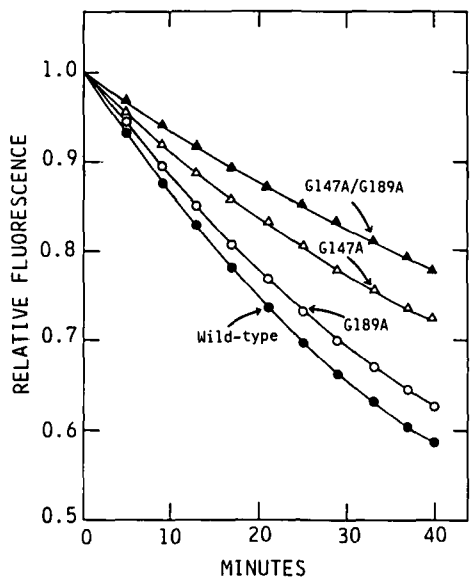

Fig. 6. Kinetics of the thermal denaturation/unfolding of NP from fluorescence emission measurements. The protein was dissolved (4 μg/ml) in 10 mM Mops buffer, pH 7.5, containing 10 mM of both NaCl and $CaCl_2$ and the sample heated at 58°C in a thermostatted cuvette. The intensity of fluorescence emission at 320 nm upon excitation at 295 nm was recorded. ●, wild-type; ○, G189A; △, G147A; ▲, G147A/G189A.

quenching of the fluorescence signal (Fontana *et al.*, 1977; Brand and Witholt, 1974), whereas a major thermal transition is seen between 55 and 75°C with a dramatic drop in fluorescence intensity. The midpoint of the transition (T_m) occurs at a lower temperature with the wild-type NP with respect to the double mutant. The values for T_m reported in Table II show clearly that the Gly to Ala replacement leads to enhancement of protein stability and, moreover, the mutational effects are cumulative.

The ranking of thermal stability of G147A/G189A > G147A > G189A > wild-type was also demonstrated by a kinetic experiment of enzyme inactivation/unfolding using fluorescence measurements. Figure 6 shows the decay in fluorescence intensity, measured at 320 nm, of the protein samples incubated at 58°C under identical conditions. Again, the double mutant is the most resistant protein species towards heat denaturation, whereas the single mutants are less thermostable than the double mutant but more thermostable than the wild-type.

Discussion

The results of this study demonstrate that *B.subtilis* NP can be stabilized by Gly to Ala substitutions without impairing the overall 3-D structure of the protein. The extent of protein stabilization, as shown by T_m or $\Delta t_{½}$ data (see Table II), occurring with single or double mutations is in the range of those previously observed with Gly to Ala substitutions located in the helical regions of T4 lysozyme (Matthews *et al.*, 1987), the λ repressor (Hecht *et al.*, 1986), *B.stearothermophilus* NP (Imanaka *et al.*, 1986) and glyceraldehyde-3-phosphate dehydrogenase (Ganter and Plückthum, 1990). Different views have emerged from these previous studies regarding the mechanistic basis of protein stabilization. It has been proposed that the Gly to Ala substitutions stabilize a protein by entropic effects (see Introduction) (Matthews *et al.*, 1987), by enhancing the helix stability caused by the introduction of a more stabilizing amino acid (alanine) within an α-helix (Hecht *et al.*, 1986; Imanaka *et al.*, 1986) or by filling internal cavities in a protein (Ganter and Plückthum, 1990). The results of this study show that protein stabilization is not restricted to replacements of helical glycines, because in mutant G189A the stabilizing exchange is located in a loop of the protein structure (Figure 1).

The structural characteristics of the sites of mutation, derived from the 3-D model of *B.subtilis* NP (Signor *et al.*, 1990; Biosym, 1991) are shown in Figures 7 and 8. The Gly147 to Ala substitution is located at the central helix (Figure 7) carrying the His142 and His146 residues coordinating the functional Zn ion, as well as the catalytic Glu143. Five out of six amino acid residues within a contact radius of 4 Å have the same side chain as thermolysin, while one conservative substitution occurs at position 205 (Leu in NP and Met in thermolysin). The introduction of a methyl group into the G147A mutant allows the filling of a cavity, thereby restoring an internal packing similar to that observed in the thermolysin molecule (Holmes and Matthews, 1982). The Gly189 to Ala substitution occurs in a long loop without any defined secondary structure (Figure 8). However, this site is buried within the protein and its environment is characterized by a number of hydrogen bonds and van der Waals contacts with the surrounding residues. The flanking chain segments carry most of the ligands for the two calcium ions and build up part of the substrate specificity pocket of the protease. The four amino acid residues located within a radius of 4 Å from site 189 in NP are conserved with respect to those of thermolysin, while two short deletions in *B.subtilis* NP near site 189 can be easily accommodated in the structure of the protein (Signor *et al.*, 1990). Also at site 189, the filling of a small cavity is achieved by the Gly to Ala substitution. Thus, on the basis of the models shown in Figures 7 and 8, it can be proposed that, among the possible mechanisms of protein stabilization by a Gly to Ala exchange (see above), clearly an improved packing of the protein interior seems to play a role in the NP system herewith investigated.

Comparative analyses in terms of amino acid sequences, 3-D structures of families of stable proteins derived from thermophilic bacteria and the labile proteins from mesophilic sources allowed the identification of the preferred amino acid exchanges (cold to hot) in the naturally occurring stable proteins from ther-

mophiles (Menéndez-Arias and Argos, 1989; Fontana, 1991a, b; Jaenicke, 1991a, b). A ranking of amino acid exchanges leading to protein stabilization was proposed on a statistical basis, the most frequent one being the Ala replacement of Gly residues located in helical regions (Menéndez-Arias and Argos, 1989). This stabilizing role of the Gly to Ala substitution can also be checked with the family of bacterial NP, because the amino acid sequences of the enzyme from both mesophilic and thermophilic sources are known. Indeed, whereas Gly189 is conserved in all bacterial NP presently known and sequenced, the Gly147 mesophilic and thermolabile NP (from *B.subtilis*, *B.amyloliquefaciens* and *B.mesentericus*) is replaced by Ala in the more stable enzyme from thermophiles (*B.thermoproteolyticus*, *B.stearothermophilus* and *B.caldolyticus*) (see Introduction for references). Of note, NP from the mesophile *B.cereus* also possesses the Gly147 to Ala substitution, and correspondingly the *B.cereus* NP shows an intermediate thermal stability between that of *B.subtilis* and *B.thermoproteolyticus* (thermolysin) NP (Sidler *et al.*, 1986; Pauptit *et al.*, 1988). Thus, it is interesting to observe that the Gly to Ala substitution has been successful in nature by engineering protein stability (Fontana, 1991a, b).

The heat inactivation and unfolding of NP and its mutants was found to be irreversible because of aggregation and autoproteolysis. Therefore it was not possible to derive values for the free energy of protein unfolding. The obvious results from this study is that the protein stabilization arising from the Gly to Ala substitution is cumulative. For example, in the case of the double mutant G147A/G189A the increase in ΔT_m of 3.2°C is approximately equal to the sum of ΔT_m of G147A plus that of the G189A mutant (see Table II). The cumulative nature of the mutational effects has been observed previously in a number of protein engineering studies and implies that the structure−stability effects of amino acid replacements are strictly local (Wells, 1990). On the other hand, noncumulative effects have been described and in these cases some form of interaction between the mutation sites is occurring by proximity effects or changes in protein conformation caused by mutation (Wells, 1990). Thus, the cumulative effect observed with the present system of *B.subtilis* NP indicates that there is no long-range interaction between the mutation sites (see Figure 1) and that the mutations cause very little, if any, change in the conformation of the protein. Indeed, CD and fluorescence emission spectra of both wild-type and mutant NP indicate that the overall secondary (far-UV CD) and tertiary (intrinsic fluorescence emission) structure of the protein is not changed, demonstrating that the methyl side chains of Ala can be incorporated into the folded structure of NP with little or no structural perturbation beyond the immediate vicinity of the site of mutation. This allows the interpretation of the differences in enzyme properties (stability/activity) in certain protein mutants, ascribing the changes solely to local changes around the modified sites.

The Ala replacements of Gly147 and Gly189 in *B.subtilis* NP do not impair the hydrolytic activity of the enzyme dramatically, because only the G147A mutant shows a reduced hydrolytic activity towards casein as substrate. On the other hand, the mutations have a varied effect on the kinetic constants of the enzyme determined using synthetic peptides as substrates (Table I). A quantitative analysis, of the values of K_m, k_{cat} and k_{cat}/K_m observed with both wild-type and mutant NP, is not simple because different peptide substrates are hydrolysed with different catalytic efficiencies. Nevertheless, on the basis of the mechanism of action of thermolysin (Colman *et al.*, 1972) and the structure of *B.subtilis* NP produced by model building (Signor *et al.*, 1990), a possible explanation of the effects of mutations at both sites 147 and 189 on the hydrolytic function of the enzyme can be attempted.

Both mutation sites are located in the proximity of the active site, but the distance of these mutation sites from the catalytic Zn ion is ~11 Å and no contacts can be detected with residues directly involved in the hydrolytic reaction (Matthews, 1989). On the other hand, the Ala side chain in position 147 (Figure 7) makes a contact with the indole group of Trp115. This residue is located in a stretch of a β-strand that helps to bind various inhibitors of thermolysin (Matthews, 1989) and presumably stabilizes the transition state of the cleavage reaction (Hangauer *et al.*, 1984). Thus, it is feasible to forecast an indirect effect of the Gly147 to Ala substitution on the S1 specificity subsite of the enzyme. Similarly, an effect of the Gly189 to Ala replacement on the specificity subsite S1 is predictable, where the hydrophobic pocket appears to be slightly reduced in size by the introduction of a methyl group.

A partial interpretation of the kinetic data collected in Table I can be proposed. The ~10-fold increase in K_m value for mutant G147A can be explained by considering a relative destabilization of the Michaelis complex, possibly caused by an interference of the newly introduced methyl side chain with the pattern of hydrogen bonds between the substrate backbone and the facing side chain residues of segment 112−115. Moreover, the reduced effect on the K_m for Z−Gly−Leu−Ala−OH can be accounted for by the lack of the side chain required for the specific interaction with the S1 subsite of the enzyme and consequently by the enhanced flexibility of this peptide substrate. As for the effects of the Gly189 to Ala mutation, a possible improvement in the binding of the P1′ Leu side chain can be proposed, corresponding to the observed loss of preference for hydrophobic side chains located at the P1 subsite. Indeed, a slightly different orientation of the peptide substrate relative to the active site may result in a balance between stabilizing factors at the S1′ subsite and destabilizing factors at S1. This could explain the observed similarity of the K_m values with the four peptide substrates analysed (Table I).

In conclusion, the results of this study, and of previous ones, indicate that the Gly to Ala substitution can be a useful strategy to produce genetically more stable proteins. Even if strategies for improving protein stability depend on the individuality of the protein (Alber, 1989; Wells, 1990; Fontana, 1991b), the cumulative mutational effects demonstrated seem to indicate the feasibility of producing enzyme mutants of a desired stability in a particular chemical or physical environment (Wetzel, 1987; Nosoh and Sekiguchi, 1990).

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