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Grafting of a Calcium-Binding Loop of Thermolysin to *Bacillus subtilis* Neutral Protease

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ABSTRACT: The surface loop which in the *Bacillus subtilis* neutral protease (NP) extends from amino acid residue 188 to residue 194 was replaced, by site-directed mutagenesis, with the 10-residue segment which in the homologous polypeptide chain of thermolysin (TLN) binds calcium-4 [Matthews, B. W., Weaver, L. H., & Kester, W. R. (1974) *J. Biol. Chem. 249*, 8030-8044]. The mutant NP was isolated to homogeneity, and its structural, functional, calcium-binding, and stability properties were investigated. Proteolytic fragmentation with *Staphylococcus aureus* V8 protease of mutant NP was used to isolate and analyze the protein fragment encompassing the site of mutation, unambiguously establishing the effective insertion of the new 10-residue segment. Atomic absorption measurements allowed us to demonstrate that mutant NP binds three calcium ions instead of the two ions bound to wild-type NP, showing that indeed the chain segment grafted from TLN to NP maintains its calcium-binding properties. The mutant NP showed kinetic parameters essentially similar to those of the wild-type NP with Z-Phe-Leu-Ala-OH as substrate. The enzyme inactivation of mutant vs wild-type NP was studied as a function of free [Ca^{2+}]. It was found that mutant NP was much less stable than the wild-type NP when enzyme solutions were dialyzed at neutral pH in the presence of [Ca^{2+}] below 10^{-3} M. On the other hand, the kinetic thermal stability to irreversible inactivation of mutant NP, when measured in the presence of 0.1 M $CaCl_2$, was found to be increased about 2-fold over that of the wild-type NP. Thus, modulation of enzyme stability by free [Ca^{2+}] in mutant NP correlates with similar findings previously reported for thermolysin. Overall, the results obtained indicate that protein engineering experiments can be used to prepare hybrid proteins on the basis of sequence and function analysis of homologous protein molecules and show the feasibility of engineering metal ion binding sites into proteins.

An exciting potential application of protein engineering is the creation of proteins with novel functions (Oxender & Fox, 1987). Although a full extension of this technology to the de novo protein design will be possible only when a deep understanding of protein folding and structure is reached (Richards, 1986), the construction of hybrid proteins represents a first promising attempt. Hybrid proteins are those obtained when two proteins, or parts of them, are joined together to give new polypeptide structures with functions eventually in common with those of the parent molecules. The first hybrid proteins reported in the literature were obtained in a more or less empirical approach, namely, by randomly fusing the DNA coding sequences of two proteins, some of which were, for example, β-galactosidase (Weinstock et al., 1983), phosphatase (Hoffman & Wright, 1985), Gal4 repressor (Fields & Song, 1989), and others. Recently, more rational approaches to hybrid protein construction have been undertaken. For example, hybrid human-mouse antibodies were created by substituting the complementarity-determining regions of a mouse antibody with the corresponding one of a human myeloma protein (Jones et al., 1986). The feasibility of substituting one β-turn for another without substantially altering the overall protein structure was demonstrated by Hynes et al. (1989). These authors prepared a hybrid staphylococcal nuclease with a β-turn of four amino acid residues replaced with a five-residue turn from concanavalin A. A crystallographic study of the mutant nuclease revealed that the con-

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formation of the guest turn segment was preserved in the hybrid protein.

We here report the feasibility of engineering hybrid proteins with specific functions, taking advantage of the knowledge of structural and functional properties of two homologous proteins. The target protein of these studies was the neutral protease (NP)[1] from *Bacillus subtilis*. This protein has been already successfully cloned and expressed by us (Toma et al., 1986), and thus methods are available for detailed investigations into structure–function relationships of NP by changing its structure according to site-directed mutagenesis methods. Indeed, initial results of protein engineering experiments have been reported (Toma et al., 1989). The *B. subtilis* NP belongs to a group of bacterial metalloendopeptidases which share many functional properties in common, such as optimum of pH, binding of zinc and calcium ions, inhibition by ion chelating agents, and similar molecular mass of 33–35 KDa. The amino acid sequences of NP from *B. subtilis* (Yang et al., 1984), as well as from *Bacillus thermoproteolyticus* (thermolysin) (Titani et al., 1972), *Bacillus stearothermophilus* (Fujii et al., 1983; Takagi et al., 1985; Kubo & Imanaka, 1988), *Bacillus cereus* (Sidler et al., 1986), and *Bacillus amyloliquefaciens* (Vasantha et al., 1984), have been determined, establishing that they share extensive homologies. Moreover, the crystal structure of thermolysin (Matthews et al., 1972, 1974; Colman et al., 1972; Holmes & Matthews, 1982) and of NP from *B. cereus* (Pauptit et al., 1988) have been solved. Thus, this set of well-characterized homologous NP proteins from thermophilic and mesophilic sources constitutes an excellent protein model system to address problems of structure–function–stability properties and relationships in proteins.

A rational exploitation of protein engineering experiments requires a detailed knowledge of both amino acid sequence and three-dimensional structure of the target protein. To this aim, we have proposed a model of *B. subtilis* NP predicted by computer graphics methods, taking as a basis the structure of the homologous thermolysin molecule (Signor et al., 1990). As expected from the ~50% homology between the two proteases (see Figure 1), the overall folding of the *B. subtilis* NP was found to be quite similar to that of thermolysin (Matthews et al., 1972, 1974; Holmes & Matthews, 1982), including the two-domain topology and gross location of elements of secondary structure. However, sequence differences and some insertions and deletions lead us to predict significant differences at protein loop regions. Moreover, whereas the catalytically important amino acid residues and those binding the functional zinc ion were conserved at the specific location in the NP chain, only the residues involved in the binding of the double calcium (Ca-1,2) in TLN are also present in NP. In fact, the carboxylate groups of Asp^{57} and Asp^{200} which bind Ca-3 and Ca-4, respectively, in TLN (Matthews et al., 1974; Roche & Voordouw, 1978) are missing (see Figure 1). A conspicuous difference in sequence between NP and TLN, as well as a deletion, is seen at the level of the "Ω loop"

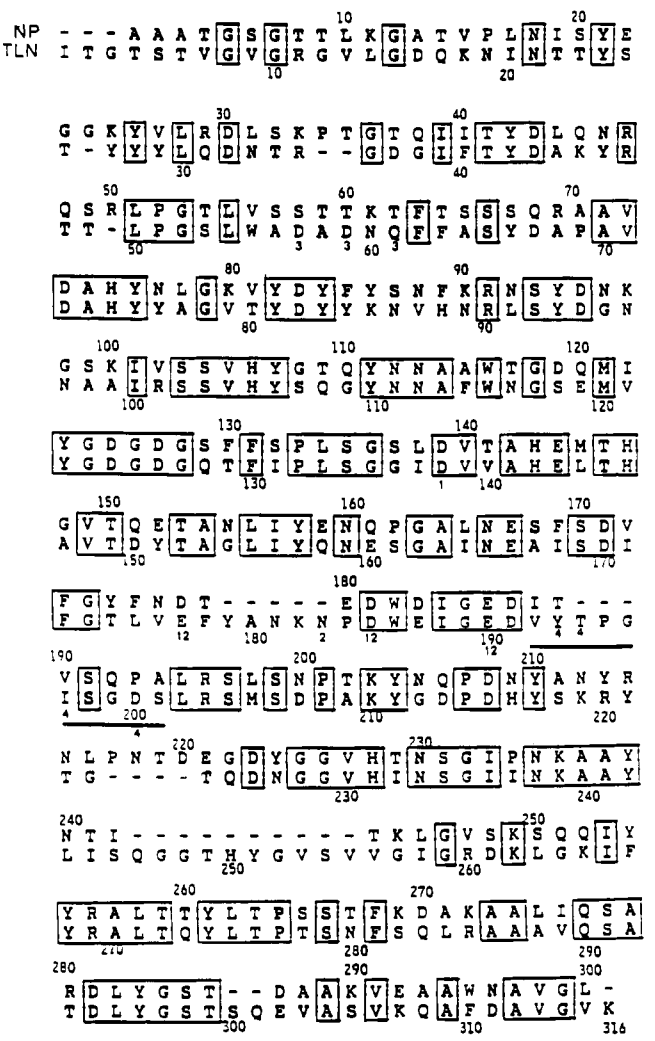

FIGURE 1: Aligned amino acid sequences of *B. subtilis* neutral protease (NP) and thermolysin (TLN) (top and bottom, respectively). Identical residues are boxed. The amino acid "ω loop" (Leszczynski & Rose, 1986) of TLN which has been used to replace the corresponding seven amino acid loop of NP is underlined. The TLN residues which provide the coordination sites for the binding of calcium ions 1–4 (Matthews et al., 1974; Roche & Voordouw, 1978) are indicated with numbers (small letters) at the bottom of the corresponding amino acid residues.

(Leszczynski & Rose, 1986) located at chain region 190–200, which binds Ca-4 in TLN. The corresponding chain segment in NP was predicted to adopt a loop conformation without resemblance to the ω loop of TLN, whereas the chain segments preceding and following this last were predicted to be of quite similar conformation in both protein molecules (Signor et al., 1990).

In this study, we have chosen the ω loop which binds Ca-4 on TLN to test the feasibility of transferring a metal binding site from TLN to NP. To this aim, a seven amino acid residue segment of NP (region 188–194; see Figure 1) was replaced by site-directed mutagenesis with the ten-residue segment of TLN (region 192–201). The mutant NP was isolated to homogeneity and characterized in terms of functional properties and stability against autolytic digestion. It is shown that the TLN loop maintains calcium-binding properties when inserted into the host protein, showing the feasibility of engineering metal ion binding sites into proteins (Pantoliano et al., 1988).

Experimental Procedures

Materials. The WT neutral protease (NP) utilized in this study was the one expressed by *B. subtilis* SMS108 cells

[1] Abbreviations: NP, *Bacillus subtilis* neutral protease; mutant NP, NP with chain segment 188–194 (seven amino acid residues) substituted with that of the homologous thermolysin chain (ten residues); WT, wild type; TLN, thermolysin; CD, circular dichroism; RP-HPLC, reverse-phase high-performance liquid chromatography; FAGLA, N^{α}-(furylacryloyl)glycylleucinamide; Gdn·HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; Mops, 4-morpholinepropanesulfonic acid; EDTA, sodium salt of ethylenediaminetetraacetic acid; $t_{1/2}$, half-life of denaturation for a kinetic experiment.

harboring plasmid pSM127 (Toma et al., 1986). The enzyme was produced and purified to homogeneity by chromatography as previously described (Toma et al., 1986, 1989; Signor et al., 1990). Thermolysin from *B. thermoproteolyticus* (Rokko) was obtained from Sigma Chemical Co. (St. Louis, MO) as a lyophilized product containing 30% calcium and sodium acetate and further purified by affinity chromatography using Sepharose–Gly-D-Phe (Pangburn et al., 1973; Walsh et al., 1974). Both purified WT NP and thermolysin were stored at −20 °C in 20 mM Tris-HCl buffer, pH 7.2, containing 10 mM $CaCl_2$. The Glu-specific protease from *Staphylococcus aureus* V8 (Drapeau, 1977) was obtained from Miles (Elkhart, IN).

Tris and Mops were obtained from Fluka (Basle, Switzerland) and the materials used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) from Bio-Rad (Richmond, CA). Ultrapure guanidine hydrochloride (Gdn·HCl) and trifluoroacetic acid (TFA) (Sequanal grade) were purchased from Pierce Chemical Co. (Rockford, IL). Solvents for reverse-phase high-performance liquid chromatography (RP-HPLC) were obtained from Merck (Darmstadt, FRG). The proteolytic substrates N^{α}-(furylacryloyl)glycyl-L-leucinamide (FAGLA) (Feder, 1968) and the synthetic peptide Z-Phe-Leu-Ala-OH (Morgan & Fruton, 1978) were obtained from Sigma and Bachem (Budendorf, Switzerland), respectively. Sephadex G-100SF and DEAE-Sephadex A-25 were purchased from Pharmacia (Uppsala, Sweden). All other chemicals and reagents were of analytical grade obtained from C. Erba (Milan, Italy) or Merck.

Preparation of Mutant Neutral Protease. The gene encoding mutant NP was prepared by oligonucleotide-directed mutagenesis following the strategy already described (Toma et al., 1989). Briefly, the *Hin*dIII–*Bam*HI fragment of the neutral protease gene containing the region to be mutagenized was isolated from plasmid pSM127 and ligated to the M13mp18 vector. The single-stranded form of the recombinant phage was then isolated and annealed to the 69-nucleotide mutagenic primer 5′-GACATCGGTGAAGAC-GTCTATACACCTGGTATTTCTGGAGATAGCCTT-AGATCTCTGTCCAACCCTACA-3′. After mutagenesis, which was verified by DNA sequence analysis (Sanger et al., 1977), the mutated *Hin*dIII–*Bam*HI fragment was introduced back into pSM127, giving plasmid pSM256. This plasmid was used to transform *B. subtilis* SMS108 strain, which then was grown for production of mutant NP following essentially the procedure described previously (Toma et al., 1989). 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 after centrifugation (8000 rpm). The precipitate was redissolved in 5 mM calcium acetate buffer, pH 6.0, and 0.1 M NaCl and dialyzed against the same buffer. The mutant NP was purified to homogeneity by three chromatographic steps of ion exchange (DEAE-Sephadex A-25), gel filtration (Sephadex G-100SF), and affinity (Sepharose–Gly-D-Phe) chromatography, in analogy with the procedure already described for the WT enzyme (Toma et al., 1986, 1989).

Analytical Methods. SDS–PAGE was carried out on a vertical slab gel (12.5%) apparatus (Laemmli, 1970). The gels (1-mm thickness) were stained with Coomassie brilliant blue R-250.

RP-HPLC analyses of WT and mutant NP and their proteolytic fragments obtained by V8 protease digestion were performed on an HPLC system obtained from LKB consisting of two Model 2150 pumps, a Model 2152 HPLC controller, an ultraviolet detector (Uvicord SD Model 2158), and a two-pen recorder (Model 2210). The instrument was equipped with a Rheodyne injection valve with injection loops of different sizes. The Aquapore RP-300 C_8 column was purchased from Brownlee Laboratories (Santa Clara, CA) and the Vydac C_{18} derivatized silica (218-TP 5415) column from Separations Group (Hesperia, CA).

The kinetic analysis of the proteolysis of the synthetic peptide Z-Phe-Leu-Ala-OH was carried out by utilizing a Perkin-Elmer HPLC instrument, Model LC-400, equipped with a Model LC-85B detector and a Varian data processor, Model 4290.

Metal ion analysis was carried out by using a Varian Model AA40 atomic absorption spectrometer.

Amino acid analyses were performed by using the phenyl isothiocyanate reagent on the Millipore-Waters Pico-tag work station and the Pico-tag column (3.9 × 150 mm) connected to a Perkin-Elmer Model LC-410 liquid chromatograph equipped with a variable detector (LC-95) and a data processor system (Model 7700) utilizing the computer program Chrom-3. Lyophilized samples of protein or peptide material (50–1000 pmol), contained in heat-treated borosilicate tubes (4 × 50 mm), were acid-hydrolyzed on the Pico-tag work station for 60 min at 150 °C using 200 μL of 6 N HCl containing 0.1% phenol. Some hydrolysates were also analyzed on a C. Erba (Milan, Italy) Model 3A29 amino acid analyzer using the single column methodology, according to the manufacturer's instructions. Tryptophan in protein and protein fragment samples was analyzed qualitatively by recording fluorescence emission spectra of solutions in 6 M Gdn·HCl upon excitation at 295 nm and observing emission near 350 nm (Brand & Witholt, 1967).

Peptide Mapping. The generation of peptide fragments from both WT and mutant NP was performed by using the Glu-specific *S. aureus* V8 protease (Drapeau, 1977). The protein sample (~0.1 mg) was dissolved in 100 μL of ammonium bicarbonate buffer, pH 7.8, containing 0.2% SDS, and then 10 μL of a V8 protease solution (2 mg/mL in acetate buffer, pH 6.0) was added. Proteolysis was allowed to proceed for 24 h at room temperature and terminated by adding to the proteolysis mixture 20 μL of 50% aqueous TFA. To the sample was added 6 μL of 7 M Gdn·HCl, and the precipitate was separated by centrifugation. This procedure was found useful for effectively removing SDS from the sample solution. The clarified solution was lyophilized on the Speed-Vac Concentrator (Savant, Hicksville, NY) and redissolved in aqueous TFA for RP-HPLC analysis.

Protein Concentration. The concentration of both WT and mutant NP were determined from absorbance measurements at 280 nm on the basis of their tryptophan and tyrosine content and by using molar absorption coefficients at 280 nm of 5690 and 1280 M^{-1} cm^{-1} for tryptophan and tyrosine, respectively (Edelhoch, 1967). The calculated $A_{280}^{1\%}$ values for WT and mutant NP were 13.8 and 14.1, respectively. For the determination of the thermolysin concentration, a value for $A_{280}^{1\%}$ of 15.2 (Voordouw & Roche, 1974) was employed. The molar concentration of proteins was calculated on the basis of a molecular weight of 33 000 for both WT and mutant NP and 34 600 for thermolysin (Titani et al., 1972).

Spectroscopic Measurements. Circular dichroism (CD) measurements were made on a JASCO Model J-500A spectropolarimeter connected to a JASCO DP-501N data processor. Rectangular cuvettes of 1-mm path length were placed in a thermostated cell holder, and the temperature inside the cell was controlled with a Haake circulating bath and mon-

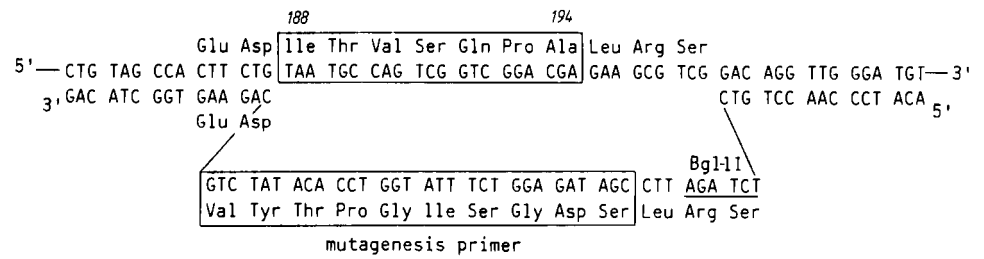

FIGURE 2: Mutation of the chain segment 188–194 of *B. subtilis* neutral protease (NP). The hybridization between the 69-nucleotide mutagenic primer (lower sequence) and the single-stranded DNA of the NP gene (upper sequence) is shown. Corresponding sequences in WT (upper) and mutant (lower) NP are boxed. The *Bgl*II site inserted into the mutated sequence is underlined (see also text).

itored by a thermistor probe (Vita et al., 1979). Fluorescence emission measurements were performed on a Perkin-Elmer Model MPF-2A or MPF-66 spectrofluorimeter (Fontana et al., 1977; Grandi et al., 1980). Spectrophotometric measurements were carried out with a Perkin-Elmer Model Lambda 15 spectrophotometer.

Metal Ion Determination. The binding of calcium to both WT and mutant NP was determined by gel filtration using a similar procedure to that described by Voordouw and Roche (1974). The enzyme was dissolved (40 mg/mL) in 20 mM Tris-HCl buffer, pH 8.0, and 5 mM $CaCl_2$, and an aliquot (1–2 mL) of this solution was loaded on a Sephadex G-100SF column (1.6 × 53 cm) equilibrated and eluted with 25 mM Tris-HCl buffer, pH 8.0, containing 0.5 mM $CaCl_2$. The calcium and zinc content in the protein-containing fractions was determined by atomic absorption spectroscopy, and protein concentrations were determined by absorption measurements at 280 nm. These data allowed us to calculate moles of ions per mole of protein. The metal ion content of TLN was determined as described (Feder et al., 1971; Voordouw & Roche, 1974).

Enzyme Assays. Proteolytic activities were measured by monitoring the decrease of FAGLA absorbance at 345 nm at 25 °C (Feder, 1968). The assay solution (3 mL) contained FAGLA (1 mM) in 50 mM Tris-HCl buffer, pH 7.0, 10 mM $CaCl_2$, and 10^{-5} M $ZnCl_2$. The catalytic reaction was initiated by adding enzyme (5–50 μL), and the decrease in absorbance at 345 nm was followed as a function of time.

The kinetic parameters K_m and k_{cat} of WT and mutant NP, as well as of TLN, were determined with the synthetic peptide Z-Phe-Leu-Ala-OH as substrate (Morgan & Fruton, 1978), by following the hydrolysis of the Phe-Leu peptide bond by HPLC analysis. Reactions were conducted at 37 °C in 50 mM Tris-HCl buffer, pH 7.0, containing 2.5 mM calcium acetate. At various time intervals, aliquots (100 μL) were removed from the reaction mixture and added to a water/acetonitrile (2:1 v/v) solution (100 μL) containing 1% H_3PO_4. The mixtures were centrifuged for 5 min at 14000*g*, and an aliquot (20 μL) of the supernatant was analyzed by HPLC utilizing a Vydac C_{18} silica column (4.5 × 150 mm, particle size 10 μm). The digestion products were separated from the peptide substrate by isocratic elution (flow rate 1.5 mL/min) with 0.05% aqueous H_3PO_4 containing 5% acetonitrile, and the effluent was monitored at 220 nm. The data were quantitatively analyzed by the aid of a Varian integrator and fitted to the Michaelis–Menten model.

Stability. The effect of calcium concentration on the stability to autolytic inactivation of both WT and mutant NP was determined by dissolving (0.13 mg/mL) the enzyme in 20 mM Tris-HCl buffer, pH 7.0, containing 10 mM $CaCl_2$. Aliquots of this enzyme solution were dialyzed for 24 h at 4 °C against the same Tris buffer containing different concentrations (10^{-6}–10^{-2} M) of $CaCl_2$. Dialysis was continued for additional 24 h at 25 °C, and then the residual enzymatic activity was assayed with FAGLA as substrate. Kinetic measurements of the thermal inactivation of WT and mutant NP were determined by incubating protease samples (0.1 mg/mL) at 55 °C in 50 mM Tris-HCl buffer, pH 7.0, containing either 0.1 M $CaCl_2$ or 1 mM $CaCl_2$ and 0.2 M KCl. At intervals, aliquots were removed from the solution for measuring the residual enzymatic activity, with FAGLA as substrate. The conformational stability of both WT and mutant NP was determined by CD measurements in the far-ultraviolet region in order to monitor the protein unfolding by incubating at 62 °C protein samples dissolved (0.1 mg/mL) in 10 mM Mops buffer, pH 7.1, containing 0.1 M NaCl and 0.1 M $CaCl_2$.

RESULTS

Preparation and Isolation of Mutant B. subtilis Neutral Protease. The cloning of the NP gene was described previously (Toma et al., 1986). The entire coding sequence together with its regulatory elements (promoter, ribosome binding site, terminator) can be isolated from plasmid pSM127 as a 3 kb *Bam*HI fragment, whereas the region coding for the mature enzyme is located within the 1.5 kb *Hin*dIII–*Bam*HI fragment. This fragment was inserted into the M13mp18 vector previously cleaved with *Hin*dIII and *Bam*HI, and the single-stranded DNA (ssDNA) was purified from phage particles. A 69-base-long oligodeoxynucleotide (Figure 2) was annealed to the ssDNA, and then the in vitro polymerization and ligation reaction was carried out (Zoller & Smith, 1983). The oligonucleotide was designed to allow its hybridization between the two stretches of 15 nucleotides preceding and following the NP coding region from amino acid 188 to 197 (Figure 2) (sequence numbering of NP; see Figure 1). The internal part of the oligonucleotide was synthesized to codify for 13 amino acids, comprising the 10 residues of the chain segment 188–197 of TLN which constitutes the "ω loop" (Leszczynski & Rose, 1986) which binds Ca-4 in TLN (Matthews et al., 1974). Three codons of the mutagenesis oligonucleotide codify for the amino acid residues Leu, Arg, and Ser (i.e., for an identical stretch in both NP and TLN, segment 195–197 in NP and 202–204 in TLN). A *Bgl*II recognition site has been introduced into the oligonucleotide, which has been used for the selection of the mutated recombinant phages. From the mutated phage DNA, the *Hin*dIII–*Bam*HI fragment was isolated and used to replace the corresponding *Hin*dIII–*Bam*HI fragment of pSM127. The sequence of the mutated region in the final plasmid construct (named pSM256) was further verified by direct sequencing of plasmid DNA (Sanger et al., 1977). The expression plasmid pSM256 was introduced via DNA-mediated transformation into the NP minus *B. subtilis* strain SMS108 which was used to express the mutant NP in analogy to the WT NP (Tomas et al., 1986, 1989).

Mutant NP was purified from the supernatant of a 2-L culture medium following the same chromatographic procedure

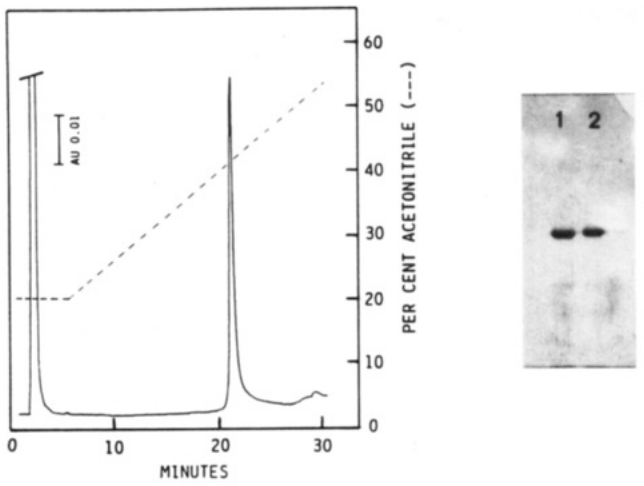

FIGURE 3: (Left) RP-HPLC analysis of mutant *B. subtilis* neutral protease (NP). A protein sample (50 μL) dissolved in 7 M Gdn·HCl solution (0.5 mg/mL) was applied to an Aquapore RP-300 C_8 column (4.6 × 100 mm), and elution was carried out at a flow rate of 0.8 mL/min with a gradient of acetonitrile in 0.05% (by volume) aqueous TFA as shown by the dashed line. The absorbance of the effluent was monitored at 226 nm. (Right) SDS–PAGE analysis of WT (1) and mutant (2) NP. Protein samples (~10 μg) purified by affinity chromatography were loaded onto 12.5% gels (Laemmli, 1970).

before described for the WT NP (Toma et al., 1986, 1989; Signor et al., 1990). Final purification was achieved by an affinity chromatographic step on Sepharose–Gly-D-Phe (Pangburn et al., 1973; Walsh et al., 1974). In analogy to WT NP, mutant NP was adsorbed onto the affinity column equilibrated with 5 mM calcium acetate buffer, pH 5.0, containing 0.1 M NaCl and then selectively eluted with 0.1 M Tris-HCl buffer, pH 9.0, containing 5 mM $CaCl_2$ (not shown). The preparations of both WT and mutant NP employed in this study were judged to be homogeneous by SDS–PAGE analysis (Figure 3). Interestingly, the mobility in the SDS gel of mutant NP is slightly lower than that of the WT species, as expected from the fact that mutant NP contains three extra amino acid residues in respect to the WT protein (see Figure 2). The homogeneity of the sample of mutant NP was established also by RP-HPLC (Figure 3). The elution time of the mutant species was indistinguishable from that of the unmodified NP.

Table I: Amino Acid Composition of Wild-Type *B. subtilis* Neutral Protease, Its Mutant, and V8 Protease Peptides[a]

amino acid	wild type	mutant	peptide 181–221[b]	peptide 187–221[b]
Asp	40.3 (42)	41.8 (43)	10.7 (11)	10.1 (10)
Glu	22.5 (22)	21.7 (21)	4.6 (4)	2.8 (2)
Ser	29.9 (30)	30.7 (31)	2.9 (3)	4.0 (4)
Gly	26.5 (26)	28.0 (28)	1.1 (1)	2.3 (2)
His	5.0 (5)	4.9 (5)	– (0)	– (0)
Arg	9.8 (9)	10.1 (9)	2.0 (2)	2.1 (2)
Thr	29.5 (30)	29.8 (30)	2.5 (3)	2.9 (3)
Ala	25.9 (26)	25.1 (25)	2.2 (2)	1.1 (1)
Pro	11.0 (11)	11.3 (11)	3.8 (4)	3.7 (4)
Tyr	21.8 (22)	22.8 (23)	3.0 (3)	4.0 (4)
Val	15.1 (15)	14.8 (15)	1.1 (1)	1.1 (1)
Met	2.0 (2)	1.9 (2)	– (0)	– (0)
Ile	10.7 (12)	11.0 (12)	1.6 (2)	0.6 (1)
Leu	20.8 (21)	21.0 (21)	3.0 (3)	2.9 (3)
Phe	9.0 (9)	9.0 (9)	– (0)	– (0)
Lys	15.1 (15)	15.0 (15)	1.2 (1)	1.0 (1)
Trp	+ (3)	+ (3)	+ (1)	(0)

[a] Values are the average results of three analyses. The amino acids are listed in the order as they elute from the Pico-tag column (see Experimental Procedures). Theoretical values (in parentheses) are those calculated from the amino acid sequence. [b] The peptide was obtained by V8 protease digestion of the WT neutral protease (NP) (peptide 181–221) and of mutant NP (peptide 187–221) and isolated to homogeneity by RP-HPLC. Peptide 187–221 contains the 10-residue segment of the thermolysin chain (see text). The sequence numbering of the peptides is that of the WT NP.

Chemical Studies. Table I shows the amino acid analyses of acid hydrolysates of WT and mutant NP. On the basis of limitations of these analyses, it is seen that the composition of WT NP is in agreement with that calculated from the amino acid sequence deduced from DNA analysis (Yang et al., 1984). On the other hand, the composition of mutant NP is that expected for the exchange of the 7-residue segment 188–194 of WT NP with the 10-residue segment of the TLN chain (see Figures 1 and 2). In fact, differences are observed for the content of those residues expected to be different in WT and mutant NP, i.e., Asp, Glu, Ser, Gly, Ala, and Tyr.

In order to more clearly substantiate that mutation had been effectively achieved, samples of both WT and mutant NP were digested with *S. aureus* V8 protease, which cleaves preferentially at Glu–X peptide bonds (Drapeau, 1977). The V8 protease digests were purified by RP-HPLC, and the elution

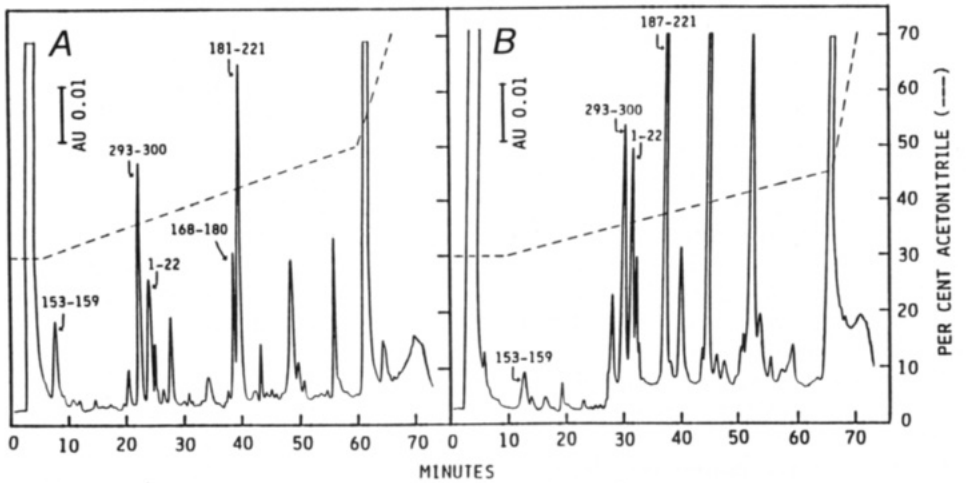

FIGURE 4: Purification of peptides obtained by V8 protease digestion of *B. subtilis* neutral protease (NP) and mutant NP by RP-HPLC. An aliquot of the proteolytic mixture (0.1 M ammonium bicarbonate, pH 7.8, containing 0.2% SDS) was applied to an Aquapore RP-300 C_8 narrow-bore column (2.1 × 100 mm), and elution was carried out at room temperature at a flow rate of 0.3 mL/min with a gradient of acetonitrile in 0.05% (by volume) aqueous TFA, as shown by the dashed line. (A) V8 digest of wild-type NP; (B) V8 digest of mutant NP. Numbers near the chromatographic peaks refer to the identity of the peptide material eluted from the column. The sequence numbering is that of wild-type *B. subtilis* NP (see Figure 1). Peptide 187–221 (part B; V8 digest of mutant NP) contains, instead of the seven-residue segment of the wild-type NP chain, the ten-residue segment of the thermolysin chain (see text).

profiles obtained are shown in Figure 4. The peptide material of the individual chromatographic peaks was collected and then acid hydrolyzed for amino acid analysis. The results of these analyses (not shown) were compared with the postulated amino acid composition of peptides expected to be produced on the basis of the location of Glu residues along the chain of both WT and mutant NP (see Figure 1), allowing the identification of the peptides listed in Figure 4. The peptide material of some chromatographic peaks did not give amino acid compositions that could be referred unambiguously to specific protein fragments, likely due to nonhomogeneity of the peptide material analyzed. Since the aim of the proteolytic fragmentation of WT and mutant NP was to isolate protein fragments encompassing the chain segment of the predicted mutation, a complete analysis of the chromatographic profiles shown in Figure 4 was not attempted. The peptides relevant for the purposes of the present study are the 41-residue fragment 181–221 and the 38-residue fragment 187–221 (sequence numbering of the WT NP chain) obtained from the V8 protease digests of WT and mutant NP, respectively, and identified on the basis of their amino acid compositions (Table I). These peptides are those expected for V8 protease cleavages at Glu residues in positions 180, 186, and 221 (see Figure 1). Of note, the Glu^{186}–Asp^{187} peptide bond in WT NP appears to be resistant to V8 protease digestion, whereas the same bond is effectively cleaved in mutant NP. This variability of the splitting of the Glu–Asp bond by V8 protease has been noted previously (Drapeau, 1977). Thus, the amino acid analyses of acid hydrolysates of the protein fragments encompassing the site of mutation, as well as those of intact WT and mutant NP (Table I), account for the expected structure of mutant NP.

Conformational Studies. A conformational analysis of both WT and mutant NP was carried out by using circular dichroism (CD) and fluorescence emission measurements (not shown). Far- and near-ultraviolet CD spectra of the two proteins (10 mM Tris-HCl buffer, pH 7.2, containing 10 mM $CaCl_2$) at 22 °C were essentially identical, indicating that the two proteins share the same overall secondary (far-UV) (Greenfield & Fasman, 1969) and tertiary (near-UV) (Strickland, 1974) structure. Fluorescence emission spectra (excitation at 295 nm) of both proteins were also similar in intensity and maximum wavelength of emission, thus showing that in both proteins the Trp residues in positions 116, 182, and 295 of the NP chain (see Figure 1) are located in a microenvironment characterized by the same polarity and degree of exposure to solvent (Brand & Witholt, 1967).

Metal Binding. The problems connected with metal ion binding and determination with proteolytic enzymes have been discussed by Voordouw and Roche (1974). Thus, the possible autolytic degradation of the protein required the development of special gel filtration procedures for analyzing both zinc and calcium content in TLN (Feder et al., 1971). In this study, in order to analyze the calcium binding of both WT and mutant NP, the gel filtration procedure described by Voordouw and Roche (1974) was employed with some modifications (see Experimental Procedures). A concentrated solution of enzyme (40 mg/mL) was loaded onto a gel filtration column equilibrated and eluted with 0.5 mM calcium containing buffer. Calcium was added to the eluting buffer in order to prevent removal of weakly bound calcium ions from the protein during the gel filtration experiment. The calcium and zinc content in the collected fractions from the column was determined by atomic absorption spectroscopy, and the molar ratios between metal and protein were determined by measuring the protein concentration. For comparison, the zinc and calcium content in TLN was analyzed following procedures already described (Feder et al., 1971; Voordouw & Roche, 1974). Table II summarizes the results obtained. As expected, 1:1 was the molar ratio between zinc and protein for the three enzymes. On the other hand, the numbers of bound calcium ions per protein molecule were 2, 3, and 4 in the case of WT NP, mutant NP, and TLN, respectively. These results lead to the conclusion that the structure of the TLN loop inserted into WT NP maintains calcium-binding properties.

Table II: Metal Ion Content of Wild-Type *B. subtilis* Neutral Protease (NP), Mutant NP, and Thermolysin[a]

enzyme	Zn (mol/mol)	Ca (mol/mol)
B. subtilis NP	1.03 ± 0.06	1.92 ± 0.05
mutant NP	0.87 ± 0.02	2.96 ± 0.18
thermolysin	1.0 ± 0.05	3.8 ± 0.1

[a] The metal content of the enzyme samples was determined by atomic absorption spectroscopy on solutions of protein samples obtained after gel filtration (see Experimental Procedures). The values given are the average results obtained by analyzing separately fractions collected from the Sephadex column. The metal stoichiometries were based on protein concentration determined by absorption measurements at 280 nm and a molecular weight of 33 000 for WT and mutant NP and 34 600 for thermolysin.

Table III: Kinetic Parameters of Wild-Type *B. subtilis* Neutral Protease (NP), Mutant NP, and Thermolysin[a]

enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
wild-type NP	1986 ± 38	1.42 ± 0.06	1395
mutant NP	1235 ± 30	1.35 ± 0.05	918
thermolysin	1462 ± 55	1.12 ± 0.11	1308

[a] Assays were conducted as described under Experimental Procedures with Ala-OH as substrate (Morgan & Fruton, 1978). The reaction medium contained 10–30 μg/mL enzyme in 50 mM Tris-HCl buffer, pH 7.0, and 2.5 mM $CaCl_2$. Hydrolysis of the substrate (i.e., cleavage at the Phe–Leu peptide bond) was followed by RP-HPLC analysis (see text). The K_m and k_{cat} were determined by fitting the data to the Michaelis–Menten model.

Functional Properties. The kinetic parameters of mutant NP were determined by using the synthetic peptide substrate Z-Phe-Leu-Ala-OH (Morgan & Fruton, 1978) and compared with those of the WT enzyme. As given in Table III, the K_m values of the mutant and WT NP are essentially identical, whereas the k_{cat} value of mutant NP is slightly lower than that of WT NP.

Stability. As expected from previous studies conducted on both *B. subtilis* NP (Voordouw et al., 1976) and thermolysin (Drucker & Borchers, 1971; Feder et al., 1971; Voordouw & Roche, 1975; Dahlquist et al., 1976), it was found that the Ca^{2+} concentration in the NP solutions had a most critical role in preventing enzyme inactivation due to autolysis. To determine the calcium dependence for the enzyme stability, solutions of both WT and mutant NP were dialyzed for 24 h at 4 °C and for an additional 24 h at 25 °C against 20 mM Tris buffer, pH 7.0, containing different Ca^{2+} concentrations, and then the residual enzymatic activity was assayed with FAGLA as substrate. The results shown in Figure 5 indicate that both enzyme species are stable when $[Ca^{2+}]$ is at least 1 mM, whereas at lower $[Ca^{2+}]$ enzyme inactivation is pronounced, especially for mutant NP. Analysis by SDS–PAGE of the dialyzed enzyme samples confirmed that the loss of enzyme activity correlated with the loss of the intact protein band (not shown). Of interest, the data of Figure 5 appear to fit a titration curve for a calcium binding site in mutant NP with an apparent log $K_a \simeq 4$.

Kinetic measurements of the thermal inactivation of WT and mutant NP were performed by incubating at 55 °C the enzyme dissolved (0.1 mg/mL) in Tris buffer, pH 7.0, and

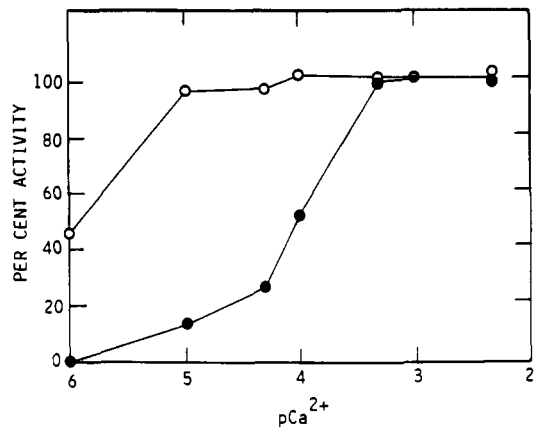

FIGURE 5: Effect of calcium concentration on the stability of WT (○) and mutant (●) neutral protease. The enzyme was dissolved (0.13 mg/mL) in 20 mM Tris-HCl buffer, pH 7.0, containing 10 mM $CaCl_2$ and then dialyzed for 24 h at 4 °C against the same Tris buffer containing different concentrations of $CaCl_2$. The dialysis was conducted for additional 24 h at 25 °C and the residual protelytic activity of the dialyzed enzyme samples assayed with FAGLA as substrate.

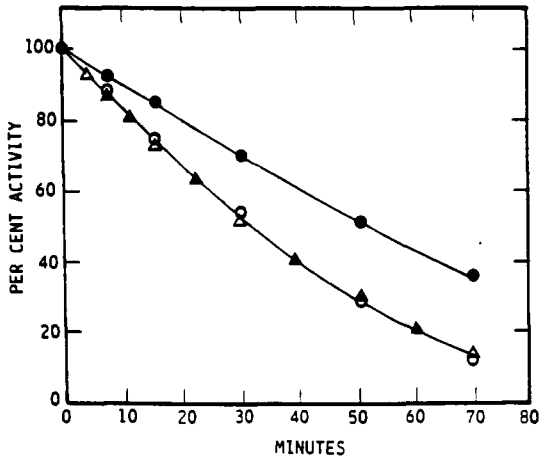

FIGURE 6: Thermal inactivation of WT and mutant neutral protease (NP). Mutant (circles) or WT (triangles) NP was dissolved (0.1 mg/mL) in 50 mM Tris-HCl buffer, pH 7.0, containing 0.1 M $CaCl_2$ or 1 mM $CaCl_2$ and 0.2 M KCl and incubated at 55 °C. At various time intervals aliquots were removed for the measurement of residual activity at 25 °C with FAGLA as substrate. (●) Mutant NP in 0.1 M $CaCl_2$; (○) mutant NP in 1 mM $CaCl_2$/0.2 M KCl; (▲) WT NP in 0.1 M $CaCl_2$; (△) WT NP in 1 mM $CaCl_2$/0.2 M KCl.

determining the residual activity in aliquots taken at intervals from the solution. As shown in Figure 5, both protein species are inactivated at the same rate in the presence of 1 mM $CaCl_2$. On the other hand, a substantial increase in thermal stability to irreversible inactivation is observed when the mutant NP is incubated in the presence of 0.1 M $CaCl_2$, whereas the stability of the unmodified enzyme is unaffected by the higher $CaCl_2$ concentration (Figure 6). The enhanced thermal stability in 0.1 M $CaCl_2$ of mutant NP is not a consequence of an increase of the ionic strength of the solution, since the stabilizing effect is not observed when the mutant NP was incubated at 55 °C in 0.2 M KCl containing 1 mM $CaCl_2$.

The results of calcium-induced stabilization of mutant NP with respect to the WT NP have been substantiated also by monitoring the thermal stability of the protein secondary structure using CD measurements in the far ultraviolet region (Greenfield & Fasman, 1989). As shown in Figure 7, the conformational stability of mutant NP, monitored by recording the dichroic signal at 220 nm, is significantly higher than that of the unmodified NP when both proteins are heated at 62

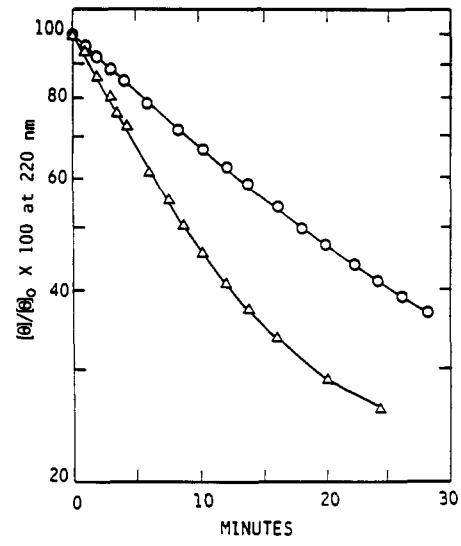

FIGURE 7: Kinetics of the thermal unfolding/denaturation of mutant (○) and WT (△) neutral protease (NP) as followed by CD measurements. The protein was dissolved (0.1 mg/mL) in 10 mM Mops buffer, pH 7.1, containing 0.1 M NaCl and 0.1 M $CaCl_2$, and the sample was heated at 62 °C in a thermostated cuvette. The time dependence of the dichroic signal at 220 nm was recorded, and the results are expressed as $[\theta]/[\theta]_0$ versus time, where $[\theta]_0$ is the initial mean residue ellipticity.

°C in Mops buffer, pH 7.1, containing 0.1 M $CaCl_2$.

The $t_{1/2}$ (half-life of denaturation) of mutant NP in the presence of 0.1 M $CaCl_2$ is roughly 2-fold higher than that of the unmodified enzyme, when the thermal stability of enzyme activity (Figure 6) or of the enzyme conformation (Figure 7) is measured. Of note, a direct comparison of the data of Figures 6 and 7 is difficult, considering that the two parameters (activity and conformation) are not necessarily strictly related and that the two experiments have been conducted under different experimental conditions of buffer composition and temperature. As expected from the data in the literature (Ohta, 1967; Khan et al., 1978; Dahlquist et al., 1976; Fujita et al., 1979; Fontana et al., 1976, 1977), TLN was fully resistant to heat denaturation when incubated for 1 h under the same experimental conditions as those used for NP (figures 6 and 7).

Discussion

This study shows the feasibility of a successful grafting of a functional loop from one protein to another, taking as a basis the knowledge of both the host and the donor protein structures. To this aim, we made use of the model for the *B. subtilis* NP three-dimensional structure recently proposed by us on the basis of the sequence homology (~50%) between NP (Yang et al., 1984) and TLN (Titani et al., 1972) and by utilizing computer graphics methods (Signor et al., 1990). The two protein chains do not show homology at the level of the Ω loop (segment 192–201) (Leszczynski & Rose, 1986) which binds Ca-4 in TLN (Matthews et al., 1974). Thus, at this site the conformation of NP is different from that of TLN, whereas the chain segments flanking this site show high similarity of both sequence (see Figure 1) and three-dimensional structure. This observation suggested that it might be possible to exchange the loop of NP (segment 188–194) with the longer one (segment 192–201) of TLN as a structural cassette (Hynes et al., 1989). On these grounds, it was predicted that the grafting of the Ω loop of TLN to the NP molecule would cause the least perturbation of the NP overall structure. The results obtained support this view and appear to substantiate the correctness of the proposed model of NP (Signor et al., 1990)

and of predictions derived therefrom.

For an easy and straightforward interpretation of the effects of a site mutation in a protein molecule, it is mandatory that the only difference between the mutant and the WT protein be located at the altered site. This has been shown to be the case for several protein mutants, as for example for T4 lysozyme mutants (Matthews et al., 1987; Matsumura et al., 1989; and references cited therein), but it may well be that it is not general, as verified for mutants of staphylococcal nuclease (Hibler et al., 1987; Wilde et al., 1988). In the present case, we made use of far- and near-ultraviolet CD as well as fluorescence emission measurements to determine features of secondary and tertiary structure of WT and mutant NP (Greenfield & Fasman, 1969; Strickland, 1974). Since these measurements did not evidence differences between the two NP species, we may conclude that grafting of the calcium-binding Ω loop to NP does not lead to appreciable alteration of the overall folding of the protein. Moreover, since protein structure is a prerequisite for function, we may recall that mutant NP maintains both enzymatic activity and efficient binding to the inhibitor Gly-D-Phe covalently bound to Sepharose. Incontrovertible proof would have been the structure elucidation of both WT and mutant NP by crystallography, but unfortunately, efforts to obtain from NP crystals suitable for X-ray analysis have been as yet unsuccessful (B. W. Matthews and M. Bolognesi, personal communication).

The hybrid NP shows functional properties similar to those of the WT NP. Slight differences between WT and mutant NP were detected when kinetic parameters were compared, with a k_{cat} value for the synthetic substraten Z-Phe-Leu-Ala-OH (Morgan & Fruton, 1978) about 30% lower in mutant NP than in WT enzyme. Such a difference can be explained considering that the loop grafting occurs in a region of the enzyme which is involved in substrate or inhibitor recognition, near the S_1 subsite (Hangauer et al., 1984; Signor et al., 1990). Thus, the NP molecule can tolerate significant sequence variability at the level of a loop without impairing its overall folding and functional properties. Actually, this is apparent also from comparative analyses of three-dimensional structures of homologous proteins (Blundell et al., 1988). Often it has been found that the "core" structures are well conserved, whereas most amino acid replacements, insertions, and deletions occur at loop regions. Moreover, recent protein engineering studies have demonstrated that loops in some proteins can be modified (Hynes et al., 1989) or even deleted (Wallace, 1987; Kuipers et al., 1989) without affecting protein function.

The Ω loop of TLN grafted to NP maintains calcium-binding properties, at least when binding is measured under the specific experimental conditions of the gel filtration technique, i.e., in the presence of 0.5 mM $CaCl_2$ (Voordouw & Roche, 1974). On the other hand, it was shown that WT NP binds only two calcium ions (see Table II), as expected from a comparative analysis of the amino acid sequences of WT NP and TLN (see the introduction). Calcium binding affinity in mutant NP can be inferred from the effect of $[Ca^{2+}]$ in preventing enzyme inactivation due to autolysis (see Figure 5). Calcium appears to bind weakly to the Ω loop in mutant NP, since the data shown in Figure 5 appear to fit a theoretical titration curve for calcium binding that has an apparent log $K_a \simeq 4$. This weak ion binding is actually in agreement with the fact that full enzyme inactivation occurs when TLN solutions are dialyzed against 10^{-6} M $CaCl_2$ (Drucker & Borchers, 1971) and TLN displays significant shelf life as well as thermal stability only when dissolved in solutions containing 1–100 mM $CaCl_2$ (Feder et al., 1971; Drucker & Borchers, 1971; Fontana et al., 1976). Moreover, previous observations indicated that Ca-4 is the most weakly bound ion to the TLN molecule. In fact, Ca-4 is easily displaced by soaking crystals of inhibited TLN in the presence of a buffer containing phosphoramidon and EDTA both at 0.1 mM concentration (Weaver et al., 1976). When an analogous experiment was performed in solution, it was found that treatment of TLN in the presence of 10 mM EDTA at pH 7.2 leads to fast enzyme inactivation as a result of an almost quantitative autolysis at peptide bonds Gly^{196}–Ile^{197} and Ser^{204}–Met^{205}, i.e., at the binding sites for Ca-4 (Fassina et al., 1986). Taking together the effect of EDTA on TLN in the crystal and in solution, it was inferred that the most easily (with millimolar EDTA) displaced calcium ion is Ca-4 and that this ion protects the corresponding ion-binding loop from autolysis (Weaver et al., 1976; Fassina et al., 1986; Fontana et al., 1986a,b). This proposal was at variance from an earlier one that the calcium ions at the double site dissociate more easily when TLN is kept in solution with free $[Ca^{2+}]$ values between 10^{-4} and 10^{-6} M (Voordouw & Roche, 1974). Thus, taking as a basis the previous observations on the binding affinity and stabilizing role of Ca-4 against autolysis in TLN, it can be proposed that in mutant NP a similar rigidification of the grafted loop upon calcium binding occurs, thus preventing enzyme inactivation due to autolysis. Moreover, the fact that mutant NP, at variance from the WT species, is maximally stabilized against thermal inactivation at 0.1 M $CaCl_2$ (see Figures 6 and 7) could be interpreted as indicating that stringent full occupancy of the weak calcium binding site in mutant NP is required to prevent autolytic inactivation at high temperature. Analogous enhancement of thermal stability of TLN was observed in the presence of 0.1 M $CaCl_2$ (Feder et al., 1971; Fujita et al., 1979), i.e., at much higher $[Ca^{2+}]$ than that required for full occupancy of the TLN calcium binding sites (10^{-3}–10^{-4} M) (Voordouw & Roche, 1974; Roche & Voordouw, 1978). In future studies, it will be of interest to study the molecular details of the autolytic degradation of WT and mutant NP under different experimental conditions and to compare these data with those already reported for TLN (Fassina et al., 1986; Fontana et al., 1986a,b).

There have been numerous studies aimed at unraveling the molecular features responsible for the unusual thermal stability of thermophilic enzymes (Zuber, 1976; Jaenicke, 1981; Fontana, 1988). Nowadays there is a general consensus that subtle structural differences between a thermophilic and a mesophilic protein are sufficient to cause thermostabilization and that a combination of the weak forces and interactions which stabilize folded proteins (hydrophobic interactions, ionic and hydrogen bonds, van der Waals interactions, metal binding, etc.) could lead to substantial thermostabilization of proteins. Binding of metal ions has been shown to play an important role in stabilizing proteins from thermophilic as well as mesophilic sources [see Pantoliano et al. (1988) and references cited therein]. The results of the present study indicate that calcium binding to a protein molecule could play a most critical role in modulating protein stability against proteolytic degradation. In the case of the homologous neutral protease family (see the introduction), one can envisage a sort of divergent evolution from an ancestral protease molecule through which stabilization/rigidification of the loop susceptible to autolysis was achieved in TLN by developing a calcium-binding property for the loop, whereas in NP, by reduction in the loop size.

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Registry No. NP, 81669-70-7; TLN, 9073-78-3; Z-Phe-Leu-Ala-OH, 24955-29-1; Ca, 7440-70-2.

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Inhibition of HIV Protease Activity by Heterodimer Formation†

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ABSTRACT: The dimeric nature of the HIV protease has been exploited to devise a novel mode of inhibiting the enzyme. The use of defective monomers or nonidentical subunits to exchange with wild-type homodimers produces catalytically defective heterodimers. Incubation of the HIV1 or HIV2 protease with a 4-fold molar excess of an inactive mutant of HIV1 leads to 80 and 95% inhibition of enzyme activity, respectively. Incubating HIV1 and HIV2 proteases at a 1:5 ratio results in a 50% reduction of activity of the mixed enzymes. The HIV1/HIV2 heterodimer was identified by ion-exchange HPLC. The heterodimer may display a disordered dimer interface, thereby affecting the catalytic potential of the enzyme. This mechanism of inactivation is an example of a dominant negative mutation that can obliterate the activity of a naturally occurring multisubunit enzyme. Furthermore, it provides an alternative to active-site-directed inhibitors for the development of antiviral agents that target the dimeric interface of the HIV protease.

The homodimeric nature of the protease from the human immunodeficiency virus 1 (HIV1)[1] has been confirmed by X-ray crystallographic (Navia et al., 1989; Wlodawer et al., 1989) and biochemical analysis (Meek et al., 1989). The two N-termini (residues 1-4 and 1′-4′) and two C-termini (residues 94-99 and 94′-99′) of the HIV1 protease dimer form β strands that interdigitate to create a four-stranded antiparallel β sheet (Wlodawer et al., 1989). This β sheet is partially stabilized by intersubunit backbone H bonds of alternate amino acids from each of the four strands (Figure 1A). Approximately 50% of the amino acid residues at the dimer interface with intermolecular distances between 2.5 and 3.3 Å are provided by this arrangement of the four termini.

Assembly of two HIV1 protease monomers results in a dimer of approximately 22000 daltons and generates an active site at the interface of the subunits. Each monomer contributes half of the active site which includes two catalytic aspartic acids as well as threonine/serine and glycine residues, which are conserved among all aspartyl proteases for their structural role in active-site geometry (Pearl & Taylor, 1987). The extensive interface that exists between the monomers is dominated by interactions between adjacent amino and carboxyl termini as well as those between twin amino acids at the active site (Wlodawer et al., 1989). Dimer formation generates not only the catalytic center but also the extended substrate binding pocket.

Viral proteolytic activity is essential for the generation of infectious virus particles in HIV and related retroviruses (Kohl et al., 1988). Autoprocessing of the protease from the *gag* and *gag/pol* polyprotein precursors results in the release of the protease and the generation of mature structural and enzymatic proteins (Debouck et al., 1987; Giam & Boros, 1988; Lillehoj et al., 1988). The pivotal role of the protease in posttranslational processing of viral proteins makes it a prime target for drug design. Agents that specifically and effectively inhibit the hydrolytic activity of the protease may serve as powerful antiviral pharmaceuticals (DesJarlais et al., 1990; McQuade et al., 1990; Meek et al., 1990). The requirement for the protease to dimerize provides an alternative mechanism for inhibiting enzyme activity than active site-directed inactivation. Prevention of the HIV protease homodimer assembly or disruption of the assembled dimer interface should effectively block viral protease catalysis. Inhibition of the heterodimeric herpes virus ribonucleotide reductase by synthetic peptides corresponding to the C-terminus of the small subunit suggests that interference in normal subunit interactions can lead to enzyme inactivation (Cohen et al., 1986; Dutia et al., 1986).

We have previously identified an autolysis product of the HIV1 protease which lacks the N-terminal five amino acids and results from the hydrolysis of a surface-accessible Leu5-

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[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HIV, human immunodeficiency virus; HPLC, high-pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

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