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Cold Adaptation of Proteins

PURIFICATION, CHARACTERIZATION, AND SEQUENCE OF THE HEAT-LABILE SUBTILISIN FROM THE ANTARCTIC PSYCHROPHILE *BACILLUS* TA41*

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The gene of subtilisin S41, an alkaline protease secreted by the psychrophile *Bacillus* TA41, encodes for a preproenzyme of 419 amino acids residues. The nucleotide sequence and NH_2- and COOH-terminal amino acid sequencing of the purified enzyme indicate that the mature subtilisin S41 is composed of 309 residues with a predicted M_r = 31,224. Subtilisin S41 shares most of its properties with mesophilic subtilisins (structure of the precursor, 52% amino acid sequence identity, alkaline pH optimum, broad specificity, Ca^{2+} binding) but is characterized by a higher specific activity on macromolecular substrate, by a shift of the optimum of activity toward low temperatures, and by a low thermal stability. The enzyme also differs by an acidic pI (5.3) and the presence of one disulfide bond. It is proposed that the psychrophilic enzyme possesses a more flexible molecular structure when compared to mesophilic and thermophilic subtilases in order to compensate for the reduction of reaction rates at low temperatures. The model of subtilisin S41 indeed reveals several features able to induce a more flexible, heat-labile conformation: the occurrence of four extended surface loops, a very hydrophilic surface through 11 extra Asp residues, and the lack of several salt bridges and aromatic-aromatic interactions. The low affinity of the Ca1 calcium binding site ($K_{d(app)}$ = 10^{-6} M), resulting possibly from one chelating side chain substitution and the stacking of Gly residues, also reflect a less compact conformation. The difference of free energy of stabilization between subtilisin S41 and a mesophilic subtilisin suggests that the balance of exo- and endothermically formed weak bonds is critical for the enzyme flexibility.

Environmental factors are key determinants in the process of adaptation and evolution of living organisms. In this respect, analysis of the structure-stability relationships in proteins from extremophiles provides valuable insights on the molecular strategies adopted in response to environmental stress such as extremes of pH, pressure, ionic strength, or temperature (Jaenicke, 1991; Di Prisco, 1991). It is anticipated that the understanding of such molecular adaptations will give access to the physical basis of the forces driving the folding of a polypeptide chain. Among the microorganisms, thermophiles have been the most widely studied in an effort to identify the structural features allowing their proteins to retain a folded conformation at elevated temperatures. These enzymes are characterized by the strengthening of one or a combination of noncovalent interactions such as salt bridges, hydrophobic and weakly polar interactions, hydrogen bonding, charge-dipole interactions in α-helices, proline residues in loops, or ion binding (Jaenicke, 1991; Fontana, 1991).

At the other end of the biological temperature scale, psychrophiles have to cope with the reduction of the enzymatic reaction rates at often sub-zero temperatures. Psychrophilic enzymes are thought to have evolved toward a high conformational flexibility, which is responsible for an increased catalytic efficiency associated with a low stability (Hochachka and Somero, 1984). The study of these enzymes is still fragmental, and the molecular basis of adaptations to low temperatures remains largely unknown. Cloning of genes from psychrophilic bacteria in *Escherichia coli* results in the expression of thermolabile recombinant enzymes active at temperatures close to 0 °C, demonstrating the intrinsic character of these properties (Feller *et al.*, 1991, 1992; Davail *et al.*, 1992; Rentier-Delrue *et al.*, 1993). In most cases, however, the analysis of psychrophilic protein sequences has been impaired by insufficient homology with their mesophilic or thermophilic counterparts. A critical step will be the availability of the refined crystallographic structure of such enzymes, which presently is lacking.

Here, we report the characterization of subtilisin S41, an alkaline protease secreted by the Antarctic psychrophile *Bacillus* TA41. This enzyme was selected because 50 subtilisin-like proteases, or subtilases, have been characterized and sequenced (Siezen *et al.*, 1991). Moreover, the three-dimensional structures of subtilisin BPN' (McPhalen and James, 1988), subtilisin Carlsberg (Bode *et al.*, 1987), subtilisin Savinase (Betzel *et al.*, 1992), thermitase (Gros *et al.*, 1989), and proteinase K (Betzel *et al.*, 1988) have been reported. The large amount of data available for subtilisins, as illustrated by over 450 site-directed mutants constructed, and their industrial importance as detergent additives make them attractive model enzymes for structure-stability studies.

EXPERIMENTAL PROCEDURES

Source and Purification of Subtilisin S41—The strain *Bacillus* sp. TA41 was isolated from sea water at the Dumont d'Urville Antarctic Station (60°40'S; 40°01'E) and was grown at 4 °C for 5 days in 6 liters of marine broth containing 5 g/liter Bacto-peptone, 1 g/liter yeast extract, 15 g/liter sea salts, pH 7.6. After centrifugation of the culture at 12,000 × *g* for 15 min, the supernatant was concentrated up to 200 ml and diafiltrated against 20 mM Tris, 1 mM $CaCl_2$, pH 8.5, using a Minitan tangential flow ultrafiltration unit (Millipore) fitted with PTGC membranes (10 kDa molecular mass limit). The sample was loaded on a phenyl-Sepharose CL-4B column (5 × 15 cm) equilibrated in the above mentioned buffer and eluted with a linear gradient of isopropanol (0–15%, 500–500 ml). Fractions containing proteolytic activity were pooled, inhibited when required by diisopropyl fluorophosphate, and dialyzed against 2 × 2 liters of 20 mM Tris, 1 mM $CaCl_2$, pH 8.5. For further experiments, the purified protease was either conditioned in the appro-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X63533.

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priate buffers by gel filtration on PD10 column (Pharmacia Biotech Inc.) or lyophilized. Subtilisin Carlsberg was from Sigma.

Enzyme Assay—Proteolytic activity was routinely assayed at 25 °C using 1 mM *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF)[1] as substrate in 50 mM Tris, 1 mM $CaCl_2$, pH 8.5. Activities toward the synthetic substrate were recorded in a thermostated Uvicon 860 Spectrophotometer (Kontron) and calculated on the basis of an extinction coefficient for *p*-nitroaniline of 8,480 M^{-1} cm^{-1} at 412 nm (DelMar *et al.*, 1979). For comparative studies, azocasein was used as a nonspecific substrate. The assay was carried out at 25 °C for 15 min in a reaction mixture containing 250 µl of 50 mM Tris, 1 mM $CaCl_2$, 200 mM NaCl, pH 8.5, 100 µl of 3% azocasein in the buffer, and 150 µl of enzyme. The reaction was stopped by addition of 500 µl of 10% trichloroacetic acid. Proteolytic activity was calculated on the basis of an extinction coefficient of 900 M^{-1} cm^{-1} at 366 nm for the chromophore.

Analytical Procedures—SDS-polyacrylamide gel electrophoresis and isoelectric focusing were run essentially as described by the supplier of the electrophoresis equipment (Hoeffer Scientific Instruments). Calcium bound to subtilisin S41 was measured using a Perkin-Elmer 303 atomic absorption spectrophotometer after gel filtration on PD10 column eluted with 25 mM NH_4HCO_3, lyophilization, and solubilization in high purity water. Activation kinetics by calcium titration was performed in 50 mM Tris, 1 mM EGTA, 1 mM AAPF, pH 8.5. The desired free Ca^{2+} concentration was set by addition of 100 mM $CaCl_2$ according to a program developed by Robertson *et al.* (1982). Sulfhydryl titration by 5,5′-dithiobis(2-nitrobenzoic acid) was made in denaturing conditions before and after reduction of the proteins with sodium borohydride (Habeeb, 1973). Active site titration was performed with *N-trans*-cinnamoylimidazole according to Bender *et al.* (1966) using an extinction coefficient of 21,000 M^{-1} cm^{-1} at 310 nm in 50 mM piperazine-HCl, 2 mM $CaCl_2$, pH 6. The energy of activation (E_a) was determined from the slope ($-E_a/R$) of Arrhenius plots, and the thermodynamic activation parameters of the proteolytic reaction were calculated according to the following equations.

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad \text{(Eq. 1)}$$

$$\Delta H^* = E_a - RT \quad \text{(Eq. 2)}$$

$$\Delta S^* = 2.303\,R(\log k_{cat} - 10.753 - \log T + E_a/2.303\,RT) \quad \text{(Eq. 3)}$$

Cleavage sites of oxidized insulin B-chain were determined by digesting the peptide with 2% subtilisin S41 at 4 °C and 20 °C for 2 h in 20 mM Tris, 1 mM $CaCl_2$, pH 8.5, for 2 h. The digest was submitted to three Edman degradation cycles, and the released 3-[4-(4′-dimethylamino-azobenzene]-2-thiohydantoin amino acids were analyzed by two-dimensional thin layer chromatography. Cloning of the subtilisin-encoding gene from *Bacillus* TA41 in *E. coli* has been described previously (Davail *et al.*, 1992). The nucleotide sequence was determined on both strands using the pGEM Single Strand System (Promega) and Sequenase (U. S. Biochemical Corp.). The NH_2-terminal amino acid sequence of the native subtilisin S41 was determined using a pulsed liquid phase protein sequenator (Applied Biosystems 477A) equipped with an on-line 120A phenylthiohydantoin analyzer. Partial amino acid sequences of a CNBr peptide (purified on a Sephadex G50 column) and of a tryptic peptide (purified by high performance liquid chromatography) were also determined. The COOH-terminal amino acid sequence was investigated by carboxypeptidase Y digestion (Klemm, 1984) of 20 nmol of purified subtilisin S41. The released amino acids were analyzed using a Dionex DC300 amino acid analyzer equipped with a Waters model 440 dual wavelength colorimeter and a high performance liquid chromatography polystyrene sulfonic column (Waters, 0.4 × 25 cm).

Molecular Modeling—Atomic coordinates were obtained from the Brookhaven Protein Data Base for subtilisin BPN′ (code 2SNI; McPhalen and James (1988)), subtilisin Carlsberg (1CSE; Bode *et al.* (1987)), and thermitase (1TEC; Gros *et al.* (1989)). Structures were superimposed using the program FRODO (Jones, 1978) on an Evans and Sutherland PS330 system. The subtilisin S41 model was constructed from the subtilisin BPN′ coordinates; insertions 42a, 42b, and 75a were built from thermitase coordinates. Amino acid replacements were generated in the low energy conformation, and bad contacts were corrected according to the superimposed models. The amino acid numbering corresponds to that of mature subtilisin BPN′.

[1] The abbreviation used is: AAPF, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide.

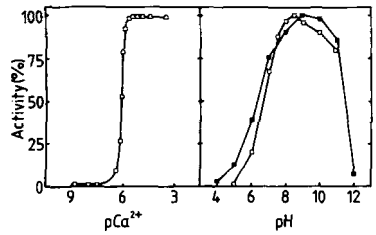

FIG. 1. **Determination of the apparent dissociation constant $K_{d(app)}$ for Ca^{2+} and of the pH optimum of subtilisin S41.** *Left*, calcium-induced activation following $CaCl_2$ titration in 20 mM Tris, 1 mM EGTA, 2 mM AAPF, pH 8.0; $pCa^{2+} = -\log[Ca^{2+}]$. *Right*, effect of pH on activity toward the macromolecular substrate azocasein (■) and the peptidic synthetic substrate AAPF (□).

RESULTS

Effect of Temperature on Growth and Protease Secretion—The strain *Bacillus* TA41 is able to grow from 0 °C to about 25 °C. However, temperatures higher than that of Antarctic sea water (−2 to 4 °C) lead to 50–60% inhibition of growth and protease secretion in the culture supernatant. Growth and alkaline protease secretion occurred concomitantly, and no sporulation or secretion stimulation in the late stationary phase was observed.

Purification and Characterization of Subtilisin S41—Alkaline protease production by *Bacillus* TA41 at 4 °C amounts to 7 mg/l and represents about 50% of the total exoproteins. A protocol based on supernatant ultrafiltration and on hydrophobic chromatography has been devised, leading to 65–70% recovery of the protease in a fairly pure state as judged by SDS-gel electrophoresis, isoelectric focusing, and limited NH_2-terminal sequencing.

The molecular mass of the purified subtilisin S41 has been estimated at 30,000 Da by gel filtration on a calibrated Sephadex G100 column. On SDS-gel electrophoresis, the apparent molecular mass is 35 kDa, but this can be related to anomalous SDS binding as reported for other subtilisins (Wells *et al.*, 1983). The calculated extinction coefficient was $\epsilon_{280} = 37{,}849$ M^{-1} cm^{-1}. The isoelectric point of the native enzyme has been found at pH 5.3. This value is drastically lower than that of mesophilic subtilisins which have pI between 9 and 11 (Markland and Smith, 1971; Betzel *et al.*, 1992). Calcium assay determined by atomic absorption indicated that S41 is a metalloprotein containing 1.5–1.8 Ca^{2+}/mol of enzyme. These figures are consistent with the low occupancy of the Ca2 calcium binding site in subtilisins (Betzel *et al.*, 1992). EGTA-mediated removal of Ca^{2+} ions results in the reversible inactivation of subtilisin S41. This allowed the determination of an apparent dissociation constant $K_{d(app)} = 1.3\ 10^{-6}$ M by activation kinetics following calcium titration (Fig. 1). This $K_{d(app)}$ value relates to the high affinity Ca^{2+} binding site because occupancy of the low affinity site (Ca2) of subtilisins is not required for activity (Pantoliano *et al.*, 1988). Like other subtilisins, the protease of *Bacillus* TA41 is relatively resistant to detergent-induced denaturation: SDS and Triton X-100 at 1% concentration result in 62% and 36% inhibition of the activity, respectively.

Substrate Specificity, Kinetic Parameters, and Temperature Dependence—The optimum pH value for activity of subtilisin S41 was found near pH 9 using either a macromolecular or a synthetic substrate (Fig. 1). It is worth mentioning that 85% of residual activity still remained at pH 11, as also reported for subtilisins Carlsberg and BPN′ (Markland and Smith, 1971).

Subtilisins have a very broad substrate specificity (Svendsen, 1976). The B-chain of oxidized insulin has been used for determining the specificity of the alkaline protease from *Bacillus* TA41. As shown in Fig. 2, subtilisin S41 has four major cleavage sites on this substrate. Hydrolysis of the bonds Gln^4-

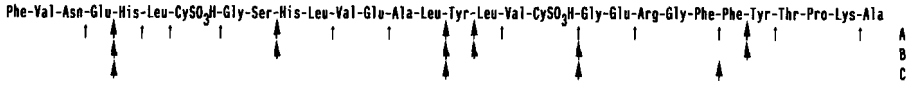

FIG. 2. **Hydrolysis of the B-chain of oxidized insulin by subtilases.** Hydrolysis by subtilisin Carlsberg (*A*) (Svendsen, 1976), subtilase Ib from *S. fradiae* (*B*), and subtilisin S41 (*C*). *Heavy arrows* indicate the main site of splitting, and *light arrows* locate bonds split after completion of the hydrolysis.

TABLE I
Relative activities of subtilisins S41 and Carlsberg toward synthetic chromogenic substrates

Substrate	Relative activity	
	Subtilisin S41	Subtilisin Carlsberg
	%	
N-Succinyl-Ala-Ala-Pro-Leu-*p*Na[a]	100	100
N-Succinyl-Ala-Ala-Pro-Phe-*p*Na	75	130
N-Succinyl-Ala-Ala-Ala-*p*Na	1.1	0.5
N-Succinyl-Gly-Gly-Phe-*p*Na	0.7	0.7
N-Succinyl-Ala-Ala-Val-*p*Na	<	<
N-Succinyl-Phe-*p*Na	<	<
N-Benzoyl-Arg-*p*Na	<	<
p-Nitrophenyl acetate	0.6	<
p-Nitrophenyl butyrate	6.0	0.9

[a] *p*Na: *p*-nitroanilide; <, not detectable.

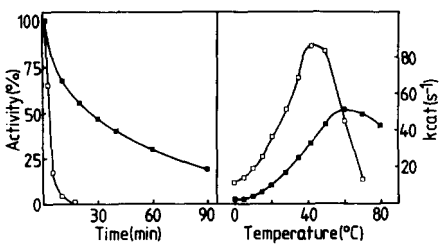

FIG. 3. **Thermal stability and thermodependence of the activity of subtilisin S41 (□) and subtilisin Carlsberg (■).** *Left*, enzymes were incubated at 50 °C in 20 mM Tris, 1 mM $CaCl_2$, pH 8.0, and residual activities of timed aliquots were recorded using AAPF as substrate. *Right*, specific activities were recorded at increasing temperatures using azocasein as substrate.

TABLE II
Kinetic and thermodynamic activation parameters of proteolysis at 15 °C

Parameter	Subtilisin S41	Subtilisin Carlsberg
k_{cat} (s^{-1})	25.4 ± 0.3 (3)	5.4 ± 0.1 (3)
E_a (kJ mol^{-1})[a]	38.5 ± 1 (16)	48.5 ± 1 (14)
ΔG^* (kJ mol^{-1})	62 ± 1	66 ± 1
ΔH^* (kJ mol^{-1})	36 ± 1	46 ± 1
ΔS^* (J mol^{-1} K^{-1})	−92 ± 3	−70 ± 2

[a] Experimental energy of activation below 15 °C.

His[5] and Leu[15]-Tyr[16] corresponds to major cleavage sites of mesophilic subtilisins, whereas splitting of bonds Cys[19]-Gly[20] and Phe[24]-Phe[25] by these enzymes is only reached after completion of the hydrolysis. The cleavage specificity of subtilisin S41 is also very close to that of protease Ib from *Streptomyces fradiae*. Increasing the incubation temperature from 4 °C to 20 °C does not modify the specificity of the psychrophilic enzyme. The side chain specificity of subtilisin S41 for the hydrolysis of short synthetic substrates does not differ markedly from the pattern of subtilisin Carlsberg (Table I). Subtilisin S41 also requires a proline residue in position P2 for optimal activity but an aliphatic residue (Leu) in position P1 leads to faster hydrolysis than with an aromatic side chain (Phe) at that position. However, the AAPF/L peptides, originally designed for chymotrypsin assay (DelMar *et al.*, 1979), are relatively poor substrates for subtilisin S41, which displays 60–70% lower k_{cat}/K_m values than subtilisin Carlsberg between 5 °C and 25 °C.

The denaturation curves of subtilisins S41 and Carlsberg

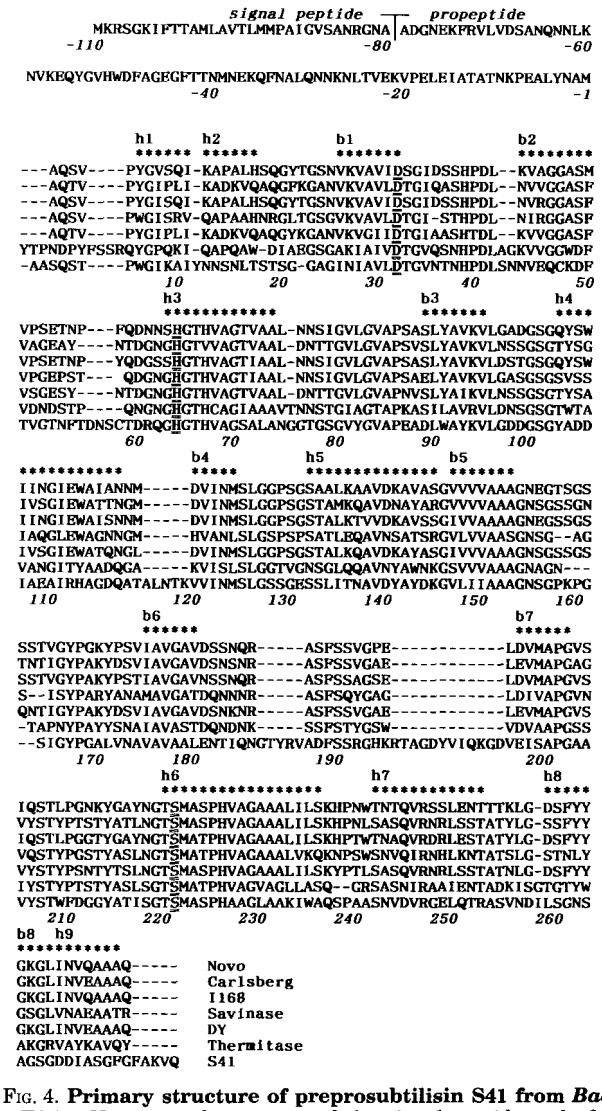

FIG. 4. **Primary structure of preprosubtilisin S41 from *Bacillus* TA41.** *Upper panel*, structure of the signal peptide and of the propeptide derived from amino acid and nucleotide sequences. Amino acids are *numbered* from the first residue of the mature enzyme. *Lower panel*, multiple amino acid sequence alignment of subtilisin BPN′ from *Bacillus amyloliquefaciens* (Wells *et al.*, 1983), subtilisin Carlsberg from *B. licheniformis* (Jacobs *et al.*, 1985), subtilisin I168 from *Bacillus subtilis* 168 (Stahl and Ferrari, 1984), subtilisin Savinase from *Bacillus lentus* (Betzel *et al.*, 1992), subtilisin DY from *B. subtilis* DY (Nedkov *et al.*, 1983), thermitase from *Thermoactinomyces vulgaris* (Meloun *et al.*, 1985), and subtilisin S41 from *Bacillus* TA41. Numbering and secondary structures correspond to the reference enzyme, subtilisin BPN′. Active site residues Asp^{32}, His^{64}, and Ser^{221} are *underlined*.

(Fig. 3) clearly illustrate the low thermal stability of the psychrophilic enzyme. The half-time of inactivation (t_i) of subtilisin S41 at 50 °C is 10 times lower when compared with the mesophilic enzyme. The difference in free energy of stabilization between both enzymes, estimated by the relation $\Delta\Delta G = 2.3RT\log(t_{i1}/t_{i2})$, amounts to 6–7 kJ mol^{-1}. The thermodependence curves of the proteolytic activity of the psychrophilic and the mesophilic enzymes are shown in Fig. 3. One can observe a shift of the apparent optimal temperature of activity of about

FIG. 5. **Stereo diagram of the α-carbon tracing of the subtilisin S41 model.** The positions of the active site side chains residues are indicated (Asp^{32}, His^{64}, and Ser^{221}). The main features of the subtilisin S41 molecule are shown. The seven amino acid residue insertions in surface loops are located by *arrows* (pointed to the first residue to be inserted). The position of Cys^{47} and of Cys^{58}, engaged in a disulfide linkage, is given, as well as the location of Thr^{77}, the Ca^{2+} coordinating ligand specific of the psychrophilic subtilisin.

TABLE III
Main structural features related to subtilisin S41 thermal lability

Parameters	S41	BPN′	Carlsberg	Savinase	Thermitase	Expected effect on S41 stability
Amino acids	309	275	274	269	279	–[a] (extended surface loops)
Asp content	21	10	9	5	13	– (hydrophilic surface)
Ionic interactions	2	5	3	7	10	–
Aromatic interactions	0	4	5	3	11	–
K_d for Ca^{2+} (M)	10^{-6}	10^{-10}	10^{-10}	?	$<10^{-10}$	–
Disulfide bond	Cys^{47}–Cys^{58}	0	0	0	0	0 (experimental)

[a] – denotes an expected decrease of stability.

20 °C toward low temperatures. Following active site titration by 1-*trans*-cinnamoylimidazole, the specific activity of subtilisin S41 toward azocasein between 5 and 20 °C was found to be 4 times higher than that of subtilisin Carlsberg and BPN′. The thermodependence curves have been used to construct Arrhenius plots and to calculate activation energy parameters of the proteolytic reaction (Table II). The lower free energy of activation (ΔG^*) of subtilisin S41 when compared with subtilisin Carlsberg correlates with its high specific activity, but the contribution of the enthalpy term (ΔH^*) and of the entropy term ($T\Delta S^*$) to ΔG^* also differs in both enzymes.

Sequence and Homology Modeling—The primary structure of the preprosubtilisin S41 deduced from nucleotide and amino acid sequences is given in Fig. 4. The potential signal peptidase cleavage site (Ala^{31}) has been located by homology with the known cleavage site of subtilisin I168 (Ala-Gln-Ala^{29} ↓ Ala-Gly-Lys) identified by Wong and Doi (1986). The cleavage site of the propeptide has been determined by NH_2-terminal sequencing of the purified mature subtilisin S41. This propeptide, composed of 79 amino acid residues in the case of prosubtilisin S41, is considered as an intramolecular chaperone characterized by two consensus sequences (Shinde and Inouye, 1993). These sequences are found between residues –57 and –47 (motif 1) and between residues –32 and –7 (motif 2). The kinetics of amino acid release by carboxypeptidase Y from the purified subtilisin S41 identified the carboxyl-terminal sequence Lys-Val-Gln-COOH and indicate that no COOH-terminal processing occurs during preprosubtilisin S41 maturation.

The mature subtilisin S41 is composed of 309 amino acid residues with a predicted M_r of 31,224. This value is somewhat higher than the mean M_r (27,000) of mesophilic subtilisins and arises from seven insertions, as shown by sequence alignment with five subtilisins and thermitase (Fig. 4). The amino acid sequence of subtilisin S41 shows 52% residue identity with the reference sequences and 71% residue similarity (G=A=S, S=T, V=L=I, F=W=Y, E=D=R=K, Q=N). Conserved amino acids occur essentially in the secondary structures of the reference enzymes and in regions bearing functional residues pertaining to the active site (Asp^{32}, His^{64}, Ser^{221}) and to the Ca^{2+} binding sites. This degree of homology allowed the building of a three-dimensional structure model of subtilisin S41 that follows the pattern of known subtilisin structures (Fig. 5).

This model was analyzed in order to identify the structural features typical of the psychrophilic enzyme. The main results are summarized in Table III. Whereas the conserved amino acid residues of subtilisin S41 pertain to the structurally conserved regions defined by Siezen *et al.* (1991), the specific insertions of the *Bacillus* TA41 enzyme belong to the variable structures of subtilases located on surface loops of the molecule. Subtilisin S41 is also characterized by a high number of charged amino acids mainly arising from 11 extra aspartic acid residues (Table III). These extra charges are located at the surface of the molecule and are most probably responsible for the anomalous isoelectric point recorded for the native enzyme. Subtilases possess conserved electrostatic and aromatic interactions involved in their folding stabilization (Teplyakov *et al.*, 1990; Siezen *et al.*, 1991). As shown in Table IV, subtilisin S41 only retains two conserved salt bridges and no aromatic interactions. The high affinity calcium binding site (Ca1) has been modelled on the basis of the known site geometry of subtilisins. Table V indicates that the calcium ligands are conserved except that Asn^{77} is replaced by Thr in the psychrophilic enzyme. The protein ligands of the low affinity calcium binding site (Ca2) are the main chain carbonyl of Gly^{169}, Leu^{171}, and Ala^{174}, whereas the positive charge of Arg^{247} stabilizes this site in the absence of the Ca^{2+} ion (Teplyakov *et al.*, 1990).

Subtilisin S41 possesses 1 cystine residue in contrast to other subtilisins. DTNB titration of the urea-denatured enzyme indeed failed to reveal any free -SH group, whereas about 2 sulfhydryl groups are detected after sodium borohydride reduction. This indicates that Cys^{47} and Cys^{58} are engaged in a disulfide linkage, which bonds the extremities of a protruding

loop between β sheet 2 and helix 3. In order to assess the influence of this disulfide bridge on subtilisin S41 stability, the native enzyme has been incubated with 0, 20, and 100 mM dithiothreitol at 45 °C (not shown). Because the activity decay of the three samples are identical, the bridge does not provide significant extra stability to the enzyme.

DISCUSSION

Subtilisin S41 secreted by the Antarctic psychrophile *Bacillus* TA41 shares most of its properties with other subtilisins from bacilli species. However, this enzyme displays a higher specific activity, a shift of the optimum of activity toward low temperatures, and a weak thermal stability, which are all common properties of cold adapted enzymes. Hochachka and Somero (1984) postulated that these enzymes are characterized by a more flexible structure allowing fast conformational changes during catalysis in order to compensate for the reduction of reaction rates at low temperatures. Thermal instability is then regarded as the consequence of the flexible structure of cold-active enzymes. The thermodynamic parameters for the enzyme-substrate complex formation (Table II) indeed agree with the fact that the activated complex will be reached through a minimum of entropy change and that less heat content would be associated with a more flexible protein structure.

All amino acids pointing their side chain toward the active site of subtilisin S41 are strictly conserved in respect to other subtilisins. This indicates that the catalytic cavity *per se* is not affected by the molecular adaptations required for catalysis at low temperatures. One can reasonably assume that slight modifications of the active site geometry will deeply alter the enzyme reaction mechanism. However, two notable amino acid substitutions in substrate binding sites at the rim of the catalytic cavity are worth mentioning. Subtilisin S41 lacks Pro^{129}, which severely restricts the mobility of a substrate binding loop in all subtilisins. Conversely, an extra proline in this loop (Pro^{131}) has been involved in the stability of savinase (Betzel *et al.*, 1992). In addition, the psychrophilic enzyme has an Ala residue at position 104, instead of an aromatic or branched aliphatic side chain, that reduces the steric hindrance at the entrance of the active site. Whereas both substitutions can be expected to lower the energy needed for conformational adjustments during catalytic events, they might be related to substrate specificity rather than to cold adaptation.

More direct evidences of structural flexibility are found at the level of subtilisin S41 conformation. As shown in Table IV, several salt bridges and aromatic interactions conserved in subtilisins are lacking within the *Bacillus* TA41 enzyme. Because the number of interactions decreases in the order thermophile > mesophile > psychrophile, there is little doubt that the removal of ion pairs and weakly polar interactions contributes to increase the flexibility of the cold-adapted enzyme. Subtilisin S41 displays four long specific amino acid insertions located in surface loops. In addition, a surprisingly large number of polar residues, mainly Asp, are found on the external shell of the protein providing a very hydrophilic surface. These two features, namely the length and the polarity of the surface loops, give rise to improved solvent interactions, reduce the compactness of the molecule, and can destabilize the psychrophilic enzyme.

Calcium contributes to the thermal stability of subtilisins by binding at specific sites and hence reduces the flexibility of the protein and its susceptibility to partial unfolding (Pantoliano *et al.*, 1988). The dissociation constant K_d of the high affinity Ca^{2+} binding site of subtilisins is close to 10^{-10} M but rises to 10^{-6} M in the case of the psychrophilic enzyme. The weak coordination of the Ca^{2+} ion is an additional insight of a less compact conformation. The predicted geometry of the high affinity calcium binding site (Table V) suggests that substitution of the conserved Asn^{77} by Thr, which can only provides coordination via the main chain carbonyl, is responsible for this effect. In addition, Thr^{77} may be less firmly oriented in the Ca^{2+} binding site as a result of the unusual stacking of Gly around this residue. On the other hand, in thermitase the replacement of the chelating side chain carbonyl oxygen of Gln^2 by a negatively charged ligand (the carboxylate oxygen of Asp^2) has been involved in the greater Ca^{2+} affinity ($K_d < 10^{-10}$ M) and stability of

TABLE IV

Electrostatic and aromatic-aromatic interactions in the psychrophilic subtilisin S41, the mesophilic subtilisins, and the thermophilic thermitase

Interacting residues	S41	BPN′	Carlsberg	Savinase	Thermitase
Electrostatic interactions					
10–184					K-D
19–271				R-E	
87/89–22/27				K-E	K-E
94–49	K-D				R-D
94–52/54				K-E	R-D
136–140		K-D	K-D		
141/145–112		K-E		R-E	
145–116					K-D
170–195		K-E	K-E		
247–197	R-E	R-D	R-E	R-D	R-D
247–251		R-E			R-E
267–184					R-D
267–255					R-D
272–255					K-D
275–271				R-E	
Aromatic-aromatic interactions					
4–206					Y-Y
4–214					Y-Y
4a–17					F-W
48–50					W-F
48–113					W-Y
50–113			F-W	F-W	F-Y
91–113		Y-W	Y-W	Y-W	
167–170					Y-Y
167–171		Y-Y	Y-Y	Y-Y	Y-Y
171–195					Y-W
192–262					Y-Y
261–262		F-Y	F-Y		
262–263		Y-Y	Y-Y		Y-W

TABLE V

Protein ligands of the high affinity calcium binding site (Ca1) in subtilases and predicted Ca^{2+} coordination in subtilisin S41

Ca^{2+} ligands are as follows: O, main chain carbonyl; $O^{\epsilon x/\delta x}$, side chain oxygen; O^{δ}, unidentate coordination by side chain carboxylate; $O^{\delta 1\delta 2}$, bidentate coordination by side chain carboxylate.

Observed				Predicted (S41)
BPN′	Carlsberg	Savinase	Thermitase	
$O^{\epsilon 1}$ Gln^2	$O^{\epsilon 1}$ Gln^2	$O^{\epsilon 1}$ Gln^2	O^{δ} Asp^2	$O^{\epsilon 1}$ Gln^2
$O^{\delta 1\delta 2}$ Asp^{41}	$O^{\delta 1\delta 2}$ Asp^{41}	$O^{\delta 1\delta 2}$ Asp^{41}	$O^{\delta 1\delta 2}$ Asp^{41}	$O^{\delta 1\delta 2}$ Asp^{41}
O Leu^{75}	O Leu^{75}	O Leu^{75}	O Val^{75}	O Asn^{75}
$O^{\delta 1}$ Asn^{77}	$O^{\delta 1}$ Asn^{77}	$O^{\delta 1}$ Asn^{77}	$O^{\delta 1}$ Asn^{77}	O Thr^{77}
O Ile^{79}	O Thr^{79}	O Ile^{79}	O Thr^{79}	O Ser^{79}
O Val^{81}	O Val^{81}	O Val^{81}	O Ile^{81}	O Val^{81}

this enzyme (Gros *et al.*, 1989).

The difference in free energy of stabilization between subtilisins S41 and its mesophilic counterpart is small (6–7 kcal mol^{-1}) and is close to the formation enthalpy of one weak bond at 37 °C. One should remember that electrostatic interactions form exothermically and are thus stabilized by a decrease of temperature, whereas hydrophobic interactions form endothermically and are destabilized at low temperature. According to these thermodynamic properties, the lack of several usually conserved salt bridges and weakly polar (aromatic) interactions in subtilisin S41 are elements that can preserve the appropriate protein flexibility at low temperatures. However, the number of weak bonds involved is probably less critical than the balance between endo- and exothermically formed interactions.

It is interesting to note that enzymes from thermophilic microorganisms reinforce the same type of weak interactions in order to gain thermal stability (Jaenicke, 1991; Fontana, 1991), showing that there is a continuum in the strategy of protein adaptation to temperature. Subtilisin S41 is therefore an appropriate candidate for site-directed mutagenesis experiments devoted to the analysis of structure-stability relationships.

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