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 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 a-helices, proline residues in loops, or ion binding (Jaenicke, 1991; Fontana, 1991).

The enzyme also differs by an acidic PI predicted to occur in the Cal calcium binding site (Kd(app) = 10^{-4} M), resulting possibly from one chelating structure of such enzymes, which presently is lacking. 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 Ca^2+ binding site, 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 Ca^2+ binding site, 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 Ca^2+ binding site, by a shift of the optimum of activity toward low temperatures, and by a low thermal stability.
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)\(^+\) as substrate in 50 mM Tris, 1 mM CaCl\(_2\), pH 8.5. Activities toward the synthetic substrate were recorded in a thermostated Uvicord 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\(_2\), 100 µl of 25% 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 determined using a Perkin-Elmer 303 atomic absorption spectrophotometer after gel filtration on PD10 column eluted with 25 mM NH\(_4\)HCO\(_3\), 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 1 mM CaCl\(_2\), according to a program developed by Robertson et al. (1982). Sulphydryl 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-cin-

The abbreviation used is: AAPF, N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide.
Right, specific activities were recorded at increasing temperatures for subtilisin Carlsberg of the hydrolysis. The cleavage specificity of subtilisin than subtilisin Carlsberg between 5 °C and 54.1 °C. Activities of timed aliquots were recorded using AAPF as substrate. Proline residue in position 174 does not modify the specificity of the psychrophilic enzyme. The half-time of inactivation of the mesophilic enzyme. The half-time of inactivation of subtilisin Carlsberg toward synthetic chromogenic substrates.

---

### Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity Subtilisin S41</th>
<th>Subtilisin Carlsberg</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Succinyl-Ala-Ala-Pro-Leu-pNa*</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>N-Succinyl-Ala-Ala-Pro-Phe-pNa</td>
<td>75</td>
<td>130</td>
</tr>
<tr>
<td>N-Succinyl-Ala-Ala-pNa</td>
<td>1.1</td>
<td>0.5</td>
</tr>
<tr>
<td>N-Succinyl-Gly-Gly-Phe-pNa</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>N-Succinyl-Ala-Ala-Val-pNa</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>N-Succinyl-Phe-pNa</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>N-Benzoyl-Arg-pNa</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>p-Nitrophenyl acetate</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>p-Nitrophenyl butyrate</td>
<td>0.6</td>
<td>0.9</td>
</tr>
</tbody>
</table>

*pNa: p-nitroanilide; <, not detectable.

---

### Table II

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Subtilisin S41</th>
<th>Subtilisin Carlsberg</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_m$ (s⁻¹)</td>
<td>25.4 ± 0.3 (3)</td>
<td>5.4 ± 0.1 (3)</td>
</tr>
<tr>
<td>$E_k$ (kJ mol⁻¹)</td>
<td>38.5 ± 1.6 (16)</td>
<td>48.5 ± 1 (14)</td>
</tr>
<tr>
<td>$\Delta G^*$ (kJ mol⁻¹)</td>
<td>62 ± 1</td>
<td>66 ± 1</td>
</tr>
<tr>
<td>$\Delta T^*$ (kJ mol⁻¹)</td>
<td>36 ± 1</td>
<td>46 ± 1</td>
</tr>
<tr>
<td>$\Delta S^*$ (J mol⁻¹ K⁻¹)</td>
<td>-92 ± 3</td>
<td>-70 ± 2</td>
</tr>
</tbody>
</table>

*Experimental energy of activation below 15 °C.

---

His⁵ and Leu¹⁶-Tyr¹⁸ corresponds to major cleavage sites of mesophilic subtilisins, whereas splitting of bonds Gys⁴⁶-Gly⁷⁰ and Phe²⁴-Phe²⁵ 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 Ibb 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 $k_m/K_m$ values than subtilisin Carlsberg between 5 °C and 25 °C.

The denaturation curves of subtilisins S41 and Carlsberg are relatively poor substrates for subtilisin S41, which displays 60–70% lower $k_m/K_m$ values than subtilisin Carlsberg between 5 °C and 25 °C.

---

**Fig. 2.** Hydrolysis of the B-chain of oxidized insulin by subtilisins. Hydrolysis by subtilisin Carlsberg (A) (Swenden, 1976), subtilase Ibb 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.

---

**Fig. 3.** Thermal stability and thermodependence of the activity of subtilisin S41 (A) and subtilisin Carlsberg (B). Left, enzymes were incubated at 50 °C in 20 mM Tris, 1 mM CaCl₂, 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.

---

**Fig. 4.** Primary structure of proproteolysis by 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 (Wolls et al., 1983), subtilisin Carlsberg from B. licheniformis (Jacobs et al., 1985), and subtilisin S41 from Bacillus TA41. Numbering and secondary structure correspond to the reference enzyme, subtilisin BPN'. Active site residues Asp²², His⁵⁶, and Ser²⁵⁰ are underlined.
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 (Asp54, His56, and Ser221). 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 Cys47 and of Cys58, engaged in a disulfide linkage, is given, as well as the location of Thr201, the Ca2+ coordinating ligand specific of the psychrophilic subtilisin.

TABLE III

<table>
<thead>
<tr>
<th>Parameters</th>
<th>S41</th>
<th>BPN’</th>
<th>Carlsberg</th>
<th>Savinase</th>
<th>Thermitase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids</td>
<td>299</td>
<td>275</td>
<td>274</td>
<td>269</td>
<td>279</td>
</tr>
<tr>
<td>Asp content</td>
<td>21</td>
<td>10</td>
<td>9</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>Ionic interactions</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Aromatic interactions</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>$K_a$ for Ca2+ (m)</td>
<td>10^{-8}</td>
<td>10^{-10}</td>
<td>10^{-10}</td>
<td>7</td>
<td>&lt;10^{-10}</td>
</tr>
<tr>
<td>Disulfide bond</td>
<td>Cys58-Cys28</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

where * - 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 ($\Delta G^*$) of subtilisin S41 when compared with subtilisin Carlsberg correlates with its high specific activity, but the contribution of the enthalpy term ($\Delta H^*$) and of the entropy term ($\Delta S^*$) to $\Delta G^*$ 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 (Ala10) has been located by homology with the known cleavage site of subtilisin I168 (Ala-Gln-Ala29; Ala-Gly-Lys) identified by Wong and Dao (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 −37 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 (Asp54, His56, Ser221) 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 subtilisases 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. Subtilisases 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 (Ca2) has been modelled on the basis of the known site geometry of subtilisins. Table V indicates that the calcium ligands are conserved except that Asn77 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 Gly169, Leu171, and Ala174, whereas the positive charge of Arg247 stabilizes this site in the absence of the Ca$^{2+}$ ion (Teplyakov et al., 1990).

Subtilisin S41 possesses 1 cysteine residue in contrast to other subtilisins. DTNB titration of the urea-denatured enzyme indeed failed to reveal any free -SH group, whereas about 2 sulphydryl groups are detected after sodium borohydride reduction. This indicates that Cys47 and Cys58 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 adaptions 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 Pro128, which severely restricts the mobility of a substrate binding loop in all subtilisins. Conversely, an extra proline in this loop (Pro113) 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 thermostable > mesophilic > psychrophilic, 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 Asn77 by Thr, which can only provides coordination via the main chain carbonyl, is responsible for this effect. In addition, Thr77 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 cheating side chain carbonyl oxygen of Gin\(^2\) by a negatively charged ligand (the carboxylate oxygen of Asp\(^5\)) has been involved in the greater Ca\(^{2+}\) affinity (\(K_d < 10^{-12}\) M) and stability of

**Table IV**

<table>
<thead>
<tr>
<th>Interacting</th>
<th>S41</th>
<th>BPN*</th>
<th>Carlsberg</th>
<th>Savinase</th>
<th>Thermitase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electric interactions</td>
<td>10-184</td>
<td>19-271</td>
<td>87/89-22/27</td>
<td>94-49</td>
<td>94-52/54</td>
</tr>
<tr>
<td>40-113</td>
<td>40-113</td>
<td>50-113</td>
<td>91-113</td>
<td>167-170</td>
<td>167-171</td>
</tr>
<tr>
<td>171-195</td>
<td>171-195</td>
<td>172-262</td>
<td>261-262</td>
<td>262-263</td>
<td>F-Y</td>
</tr>
</tbody>
</table>

**Table V**

<table>
<thead>
<tr>
<th>Protein ligands of the high affinity calcium binding site (Ca(1)) in subtilases and predicted Ca(^{2+}) coordination in subtilisin S41</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPn*</td>
</tr>
<tr>
<td>Gln(^5)</td>
</tr>
<tr>
<td>O(^1) Asp(^{11})</td>
</tr>
<tr>
<td>O Leu(^{75})</td>
</tr>
<tr>
<td>O Asn(^{77})</td>
</tr>
<tr>
<td>O Val(^{81})</td>
</tr>
</tbody>
</table>
this enzyme (Gros et al., 1989).

The difference in free energy of stabilization between subtilisin 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.

Acknowledgments—We acknowledge the “Expeditions Polaires Françaises” for support and facilities offered at the Dumont d’Urville Antarctic Station. We thank Drs. R. Cesar, J. Van Beeumen (Rijksuniversiteit Gent), O. Dideberg, and E. Fonze for valuable collaboration on these experiments and N. Gerardin-Otthiers, S. Collin, and R. Marchand for expert technical assistance.

REFERENCES


