Psychrophiles, host of permanently cold habitats, display metabolic fluxes comparable to those exhibited by mesophilic organisms at moderate temperatures. These organisms have evolved by producing, among other peculiarities, cold-active enzymes that have the properties to cope with the reduction of chemical reaction rates induced by low temperatures. The emerging picture suggests that these enzymes display a high catalytic efficiency at low temperatures through an improved flexibility of the structural components involved in the catalytic cycle, whereas other protein regions, if not implicated in catalysis, may be even more rigid than their mesophilic counterparts. In return, the increased flexibility leads to a decreased stability of psychrophilic enzymes. In order to gain further advances in the analysis of the activity/flexibility/stability concept, psychrophilic, mesophilic, and thermophilic DNA ligases have been compared by three-dimensional-modeling studies, as well as regards their activity, surface hydrophobicity, structural permeability, conformational stabilities, and irreversible thermal unfolding. These data show that the cold-adapted DNA ligase is characterized by an increased activity at low and moderate temperatures, an overall destabilization of the molecular edifice, especially at the active site, and a high conformational flexibility. The opposite trend is observed in the mesophilic and thermophilic counterparts, the latter being characterized by a reduced low temperature activity, high stability and reduced flexibility. These results strongly suggest a complex relationship between activity, flexibility and stability. In addition, they also indicate that in cold-adapted enzymes, the driving force for denaturation is a large entropy change.

The temperature range in which biological activity has been detected extends from −20 °C, the temperature recorded in the brine veins of Arctic or Antarctic sea ice (1), to 113 °C, the temperature at which the archae Pyrolobus furiosus is still able to grow (2). Although numerous investigations have been carried out on thermophilic microorganisms and on their molecular components, especially enzymes, the efforts devoted to cold-adapted microorganisms have been comparatively limited despite their tremendous biotechnological (1, 3–5) and fundamental (1, 6–8) applications. Indeed, the biochemical and physiological bases of cold adaptation, which include, for example, regulation of gene expression by low temperatures, membrane adaptation in relation with the homeoviscosity concept, and the activity/stability relationships sustaining the catalytic efficiency of cold-adapted enzymes, are still poorly understood.

In permanent cold habitats, low temperatures have constrained psychrophiles to develop among other peculiarities enzymatic tools allowing metabolic rates compatible to life that are close to those of temperate organisms. Thermal compensation in these enzymes is reached, in most cases, through a high catalytic efficiency at low and moderate temperatures (for review, see Ref. 9 and 10). The emerging picture is that is increased catalytic efficiency is attributed to an increase of the plasticity or flexibility of appropriate parts of the molecular structure in order to compensate for the lower thermal energy provided by the low temperature habitat. This plasticity would enable a good complementarity with the substrate at a low energy cost, thus explaining the high specific activity of psychrophilic enzymes. In return, this flexibility would be responsible for the weak thermal stability of cold-adapted enzymes. This relationship between activity, flexibility and stability constitutes a hot topic and represents a central issue in the adaptation of proteins to various environments. Moreover, it is believed that all proteins evolve through a balanced compromise between these features, i.e. structural rigidity allowing the retention of a specific three-dimensional-conformation at the physiological temperature and in contrast flexibility, allowing the protein to perform its catalytic function. In the context of temperature adaptation of enzymes, it is assumed that high temperatures require stable protein structure and activity, whereas high enzyme activity is mandatory at low temperatures.

While the common trait of a low conformational stability in cold-adapted enzymes has been demonstrated (see Ref. 11 for review), the stability/flexibility relationship is still controversial since some authors consider that theinstability of psychrophilic enzymes is due to a random genetic drift (12). Moreover, if the decreased stability of cold-adapted enzymes is well documented, there is however, no direct experimental evidence of an increased flexibility. Besides, controversial results were obtained when the flexibility of a few psychrophilic enzymes was investigated by measuring hydrogen-deuterium exchange rates. In the case of 3-isopropylmalate dehydrogenase (13), while the psychrophilic and mesophilic enzymes were found...
more flexible than the thermophilic counterpart, the psychrophile was however more rigid than the mesophile. Nevertheless, in this case, the technique suffered from the disadvantage of being a measure of the accessibility of deeply buried residues, and thus did not detect local flexibility, in particular that associated with the active site which is generally quite accessible. Using a similar technique, Fields (14) showed that while a psychrophilic and a mesophilic lactate dehydrogenases had similar flexibility at 2 °C, the global flexibility of the psychrophile was significantly larger at 23 °C. In addition to the lack of data about the flexibility of cold-adapted enzymes, information on the thermodynamics of inactivation and unfolding is also missing and the few available reports are controversial. Indeed, Siddiqui et al. (15) proposed that the thermoplasticity of psychrophilic enzymes is due to enthalpic effects, while others (16-18) found this to be entropically driven. Further studies are thus required to resolve whether an unfavorable entropic or enthalpic contribution determines the irreversible unfolding and inactivation of these enzymes.

In attempt to elucidate how stability, catalytic activity, and conformational flexibility are connected in psychrophilic enzymes, we have investigated three structurally homologous NAD\(^+\)-dependent DNA ligases. The psychrophilic DNA ligase from the Antarctic bacterium *Pseudoalteromonas haloplanktis* (Phig)\(^1\) has been overexpressed and characterized (19), highlighting an increased catalytic efficiency as well as an increased thermolability of the enzyme. Cold adaptation of Phig is believed to be due to a decreased level of arginine and proline residues, as well as an overall destabilization of its N-terminal domain (19). The mesophilic reference chosen is the DNA ligase form *Escherichia coli* (Eclig) (20) and the thermophilic homologue is the *Thermus scotoductus* DNA ligase (TsDig) (21). The three enzymes are of a similar size, share all the properties common to NAD\(^+\)-dependent DNA ligases, but are adapted to different extremes of the temperature scale (19), therefore constituting an adequate series of homologous enzymes for temperature adaptation studies. In the present work, the overall destabilization of Phig is examined with the help of three-dimensional modeling and investigation of solvent-exposed hydrophobic clusters. In addition, the intrinsic relationship between activity, flexibility and stability in Phig is analyzed by comparison of its temperature dependent activity, conformational flexibility, and thermal and chemical stabilities with the mesophilic and thermophilic counterpart Eclig and TsDig, respectively.

**EXPERIMENTAL PROCEDURES**

**Three-dimensional Modeling**—The target sequences for modeling are the NAD\(^+\)-dependent DNA ligases from *P. haloplanktis*, *E. coli*, and *T. scotoductus*. The best template for the three proteins was 1DGSA A, the homologue NAD\(^+\)-DNA ligase of *T. filiformis* (22). The modeling, minimizations and molecular dynamics procedures were described elsewhere.\(^2\) The equilibrium temperatures chosen, known as the optimal organism temperature, were 277, 310, and 354 K, for *P. haloplanktis*, *E. coli*, and *T. scotoductus*, respectively.

**Protein Preparation and Assays**—The recombinant cold-adapted NAD\(^+\)-dependent DNA ligase (Phig) was overexpressed at 18 °C in *E. coli* BL21(DE3) as previously described (19), except that the growth was performed in TB medium (12 g/liter tryptone, 24 g/liter yeast extract, 4 ml/liter glycerol, 12.54 g/liter KH\(_2\)PO\(_4\), 2.32 g/liter KH\(_2\)PO\(_4\), pH 7) containing 50 μg ml\(^{-1}\) kanamycin, and that induction was performed when OD\(_{600}\) reached ~4. The cold His-tagged enzyme was then purified by Ni\(^{2+}\)-Hitrap Chelating followed by thrombin digestion. The cold His-tagged enzyme was then purified by Ni\(^{2+}\)-Hitrap Chelating to separate untagged protein from the undigested ligase, and Mono Q HR 5/5, as described previously (19). Plasmid encoding *E. coli* DNA ligase was a generous gift from V. Sriskanda and S. Shimam (20). The pET-EcoLig plasmid (20) was digested with NdeI and BamHI and the insert cloned into pET22b plasmid, leading to the overexpression of an untagged protein. The recombinant Eclig was overexpressed at 37 °C in *E. coli* BL21(DE3) as previously described (20), except that the growth was performed in TB-ampicillin medium containing 100 μg ml\(^{-1}\) ampicillin, and that induction was performed when OD\(_{600}\) reached ~3. The mesophilic protein was purified according to a protocol established for *T. scotoductus* DNA ligase (TsDig) (23), except that the heatings treatments were replaced by a DEAE-agarose chromatography. The cold Eclig and TsDig was a kind gift from Z. O. Jönsen and G. Eggertsson (Reykjavik, Iceland) (21). The recombinant wild-type TsDig was overexpressed and purified by two successive heatings (65 and 80 °C), followed by Hi-Trap Heparin and Mono Q HR chromatographic steps, as described (23). Protein concentration was determined with the Coomassie Protein Assay Reagent (Pierce), using bovine serum albumin as standard. The final yield was ~100 mg, ~250 mg, and ~55 mg per liter of culture for Phig, Eclig, and TsDig respectively. N-terminal sequencing confirmed the integrity of recombinant proteins. Enzyme activity was determined using a fluorimetric assay, as described previously (19).

**Adenylation Assays**—In order to compare DNA ligases in a similar adenylation state, enzyme stock solutions were adenylated by adding an excess of β-NAD\(^+\) (24). The ratio [NAD\(^+\)/[DNA ligase]] was ~80. The adenylated mixtures were then incubated at 10 °C (Phig), 25 °C (Eclig), and 65 °C (TsDig) for 30 min and then cooled rapidly on ice. Protein solutions were dialyzed against appropriate buffer before experiments.

**Fluorescence Measurements**—Both intrinsic and ANS fluorescence measurements were recorded on an Amino SL100 spectrophotofluorimeter. Thermal denaturation (scan rate 1 °C/min) of the DNA ligases was monitored by recording the fluorescence intensity change at 330 nm, using a protein concentration of 50 μg/ml (0.66 μM) in 20 mM phosphate sodium, 50 mM NaCl, pH 7.6, with excitation at 280 nm (2-nm band pass) and emission at 330 nm (4-nm band pass). Data were normalized using the pre- and post-transition baseline slopes as described (25) and were fit according to a three-state model (26).

ANS fluorescence measurements were performed at 25 °C except for Phig (15 °C), using a protein concentration of 25 μg/ml (0.33 μM) in 20 mM phosphate sodium, 50 mM NaCl, pH 7.6, with excitation at 280 nm (2-nm band pass) and emission (4-nm band pass) spectra recorded from 420 to 600 nm (scan speed 350 nm min\(^{-1}\)). The fluorescence spectra were corrected for the background fluorescence of ANS. The ratio [ANS]/[ligase] was ~200 using ε\(_{340\text{ nm}}\) = 4950 M\(^{-1}\) cm\(^{-1}\) for ANS. Thermal unfolding of samples containing ANS was performed at a scan rate of 1 °C/min. In this case, the recorded fluorescence spectra were corrected for the background fluorescence of ANS at the corresponding temperature.

**Dynamic Quenching of Fluorescence**—The conformational residencies of Phig, Eclig, and TsDig were characterized by acrylamide-induced fluorescence quenching. Samples were prepared in 20 mM sodium phosphate buffer, 50 mM NaCl, pH 7.6, and the protein concentrations (~50 μg/ml) were adjusted to provide an optical density at the excitation wavelength less than 0.1. Aliquots of 1.2 ml acrylamide stock solution were consecutively added to 1 ml protein solution in order to increase acrylamide concentration by ~5 mM steps. Experiments were performed on an Amino SL100 spectrophotofluorimeter at 10 and 25 °C, using excitation at 280 nm with fluorescence emission set at 330 nm (stacked spectra) and emission slit widths were 1 and 4 nm, respectively, and the fluorescence intensities were recorded for 30 s. Data, that are the mean of three experiments, were corrected for the dilution effects and for the absorbive screening caused by acrylamide (ε\(_{280\text{ nm}}\) = 4.3 M\(^{-1}\) cm\(^{-1}\) for acrylamide). Quenching data were plotted as the ratio of fluorescence in the absence of quencher (F\(_0\)) to the intensity in the presence of quencher (F), the quencher concentration. The resulting data were fit to dynamic parameters according to the Stern-Volmer equation given in Equation 1.

\[
F/F_0 = 1 + K_{q}[Q] \tag{Eq. 1}
\]

where \(K_{q}\) is the Stern-Volmer quenching constant and \(Q\) the quencher concentration (27).

**Differential Scanning Calorimetry (DSC)**—Measurements were performed using a MicroCal MCS-DSC instrument as detailed (28). Samples were dialyzed overnight against 30 mM MOPS, 50 mM KCl, pH 7.6. In order to decrease aggregation, a non-detergent sulfobetaine (3-(1-
pyridinio)-1-propane sulfonate) was added prior to DSC experiment (29), to a final concentration of 0.5 M for Ph lig and Ec lig, and 0.75 M for Ts lig. Protein concentration was determined with the Coomassie Protein Assay Reagent (Pierce), and was 4 mg/ml except for Ph lig (7 mg/ml). Thermograms were analyzed according to a non-two-state model in which Tm, ΔHcal, and ΔHeff of individual transitions are fitted independently using the MicroCal Origin software (version 2.9). The magnitude and source of the errors in the Tm and enthalpies values have been discussed elsewhere (30). Fitting standard errors on a series of three DSC measurements made under the same conditions in the present study were found to be 0.1 K and 4% on both enthalpies. All scans were found to be irreversible under the experimental conditions used for these studies. Kinetically driven unfolding was recorded at two different scan rates (0.4 and 1 K min⁻¹ for Ph lig, and 1 and 2 K min⁻¹ for Ec lig), and the rate constant k_dnat was calculated from the relation shown in Equation 2 (31),

\[ k = \frac{vC_p}{\Delta H_{cal} - Q} \]  

(Eq. 2)

where v represents the scan rate (K min⁻¹), C_p the excess heat capacity at a given temperature, ΔH_cal the total heat of the process, and Q the heat evolved at the given temperature.

GdmCl-induced Unfolding—Approximately 25 μg/ml (A₂₈₀nm < 0.1) protein samples in 50 mM phosphate sodium, 50 mM NaCl, pH 7.6, were incubated overnight at 25 °C (18 °C for the cold-adapted Ph lig), in defined guanidine hydrochloride (GdmCl) concentrations, and unfolding was monitored by intrinsic fluorescence analysis. The pH was checked to ensure a constant value throughout the whole transition, and the denaturant concentration was determined from refractive index measurements (25) using a R5000 hand refractometer from Atago. Relative fluorescence was measured on an Aminco SLM 8100 spectrofluorimeter at 25 °C, except for Ph lig (18 °C), at an excitation wavelength of 280 nm (2-nm band pass) and at an emission wavelength of 330 nm (4-nm band.
pass), except Ts lig (λ<sub>ex</sub> 333 nm). Unfolding of the three DNA ligases Ph lig, Ec lig, and Ts lig was fully reversible since they regained native conformation following renaturation after complete denaturation in 7 M GdmCl. Fluorescence spectroscopy data were normalized using the pre- and post-transition baseline slopes as described (25). The transition curves obtained were analyzed using a three-state model (N→I→U) (26).

**Determination of Activation Parameters**—Thermodynamic parameters of activation were calculated as described (32), using Equations 3–5,

<table>
<thead>
<tr>
<th>Table I</th>
<th>Composition and surface accessibility of the active site (adenylation domain) of Ph lig, Ec lig, and Ts lig</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ph lig</td>
</tr>
<tr>
<td><strong>Composition</strong></td>
<td></td>
</tr>
<tr>
<td>Number of amino acids (aa) forming the active site</td>
<td>248</td>
</tr>
<tr>
<td>Number of positively charged aa (basic)</td>
<td>37 (15%)</td>
</tr>
<tr>
<td>Number of negatively charged aa (acidic)</td>
<td>35 (14%)</td>
</tr>
<tr>
<td>Number of neutral aa</td>
<td>52 (21%)</td>
</tr>
<tr>
<td>Number of nonpolar aa</td>
<td>124 (50%)</td>
</tr>
<tr>
<td><strong>Surface accessibility (Å²)</strong></td>
<td></td>
</tr>
<tr>
<td>Recalculated accessible surface</td>
<td>14375</td>
</tr>
<tr>
<td>Surface of positively charged aa (basic)</td>
<td>4339 (30%)</td>
</tr>
<tr>
<td>Surface of negatively charged aa (acidic)</td>
<td>2579 (18%)</td>
</tr>
<tr>
<td>Surface of neutral aa</td>
<td>2911 (20%)</td>
</tr>
<tr>
<td>Surface of nonpolar aa</td>
<td>4546 (32%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table II</th>
<th>Composition of the accessible surface of Ph lig, Ec lig, and Ts lig</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ph lig</td>
</tr>
<tr>
<td><strong>Surface accessibility (Å²)</strong></td>
<td></td>
</tr>
<tr>
<td>Recalculated accessible surface</td>
<td>36183</td>
</tr>
<tr>
<td>Neutral accessible surface</td>
<td>3254 (9%)</td>
</tr>
<tr>
<td>Hydrophobic accessible surface</td>
<td>7994 (22%)</td>
</tr>
<tr>
<td>Hydrophilic accessible surface</td>
<td>24935 (69%)</td>
</tr>
<tr>
<td>positively charged</td>
<td>9202 (25%)</td>
</tr>
<tr>
<td>negatively charged</td>
<td>7184 (20%)</td>
</tr>
</tbody>
</table>

**Fig. 2.** ANS fluorescence intensity. A, ANS fluorescence spectra of Ph lig, Ec lig, and Ts lig in the native state. B, temperature dependence of ANS binding by Ph lig (●) and Ec lig (○), measured by the intensity of ANS fluorescence at 480 nm (λ<sub>ex</sub> 390 nm).

**Fig. 3.** Thermodependence of activity (A) and denaturation as recorded by fluorescence emission (B). Compared with Ec lig (○) and Ts lig (●), the cold-adapted Ph lig (●) is characterized by: (i) a shift of the optimal activity toward low temperatures, (ii) an increased specific activity at low and moderate temperatures, and (iii) a low stability of its active site.
\[
\Delta G = -RT \ln \left( \frac{k_B T}{h} \right) \quad \text{(Eq. 3)}
\]
\[
\Delta H = E_a - RT \quad \text{(Eq. 4)}
\]
\[
\Delta S = \frac{\Delta H - \Delta G}{T} \quad \text{(Eq. 5)}
\]

where \( k_B \) is the Boltzmann constant (1.3805 \times 10^{-23} \text{ JK}^{-1}), \( h \) the Planck constant (6.6256 \times 10^{-34} \text{ J s}), \( k \) (s^{-1}) the rate constant at temperature \( T \) (K), \( E_a \) the activation energy of the reaction, and \( R \) (8.314 J mol^{-1} K^{-1}) the gas constant.

**RESULTS**

**Molecular Model of Adenylated Phlig, Eclig, and Tslig**—The amino acid sequences of Phlig, Eclig, and Tslig show 45, 45, and 87% identity with T. filiformis (Tf lig) DNA ligase, allowing us to build three-dimensional models from the known x-ray structure (Fig. 1, B–D). The final models of Phlig, Eclig, and Tslig can be superimposed, showing a strong conservation of the active site architecture, of the main secondary structures, and of the overall fold. These monomeric enzymes are folded into four discrete domains characterizing NAD-dependent DNA ligases (22) (Fig. 1, A and E), i.e. Domain 1 (containing Ia and adenylation subdomains), Domain 2 (OB-fold domain), Domain 3 (Zn-finger and HhH subdomains), and Domain 4 (BRCT or BRCA C-terminal-related domain). As shown for Tf DNA ligase (22), the circular arrangement of the four domains leads to a hole sufficiently large to hold a double-stranded DNA.

Sequence alignment reveals that the psychrophilic DNA ligase contains all the conserved boxes that have previously been described in eubacterial DNA ligases (Fig. 1, E). Moreover, the recent determination of the structure of Tf lig (22), as well as the investigation of Domain Ia of Eclig (33) have pointed out essential residues implicated in catalysis, that appear to be conserved among NAD-dependent DNA ligases adapted to different temperatures. To further characterize the active site of the psychrophilic enzyme, the number as well as the surface of positively charged, negatively charged, neutral, and nonpolar amino acids of the adenylation domain were determined (Table I). The main trend is a decrease in the number and mainly in the accessible surface of neutral side chains from Phlig to Eclig and Tslig, respectively.

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phobic surfaces and reduced charged surfaces when compared with the adenylating domain of the thermophilic enzyme. A similar trend is observed when the whole molecule is analyzed (Table II). As shown in Table II, the psychrophilic enzyme displays an increased exposure of hydrophobic residues to the solvent, whereas the thermophilic DNA ligase shows an increased hydrophilic accessible surface area, including an increase of the charged accessible surface. Such discrepancies probably lead to improved electrostatic interactions in the thermophilic ligase that are likely to stabilize the enzyme at high temperatures, whereas the excess of hydrophobic surfaces in Phlig represents an entropy-driven destabilizing factor.

ANS Fluorescence—It is well known that the interaction of hydrophobic fluorescent probes such as 8-anilino-1-naphtalsulfonic acid (ANS) with the exposed hydrophobic sites on the surface of native protein results in a considerable increase of the dye fluorescence intensity and a blue shift of its fluorescence spectrum (34, 35). As the three-dimensional modeling predicted an increased exposure of hydrophobic residues to the solvent in Phlig, the binding of ANS was investigated in the three adenylated DNA ligases.

As seen in Fig. 2A, no significant binding of ANS can be observed with the native adenylated Tslig. In the case of Eclig, a slight binding is detected, suggesting the exposure of some hydrophobic patches at the surface of the native enzyme. By contrast, strong enhancement of ANS fluorescence is recorded for Phlig, underlining a significant population of solvent-accessible nonpolar clusters in the native enzyme. Changes in ANS fluorescence were also monitored during thermal denaturation. In the case of Phlig (Fig. 2B), a slight increase of ANS binding is observed when increasing the temperature, which reaches a maximum around 33 °C (corresponding to a maximal surface exposition of hydrophobic clusters), and then decreases to an intensity lower than that of the native enzyme. Eclig displays a similar behavior (Fig. 2B), except that the increase in ANS fluorescence is more pronounced when increasing the temperature, reaches a maximum around 55 °C and then decreases at higher temperatures, but still exhibits significant ANS fluorescence, at a level significantly higher than that of the native enzyme. Such result suggests that Eclig is more prone to form aggregates at high temperatures. Such aggregates are still able to bind ANS, leading to the observed ANS signal at high temperatures. The high stability of Tslig (see below) did not allow monitoring ANS changes occurring upon thermal denaturation.

Thermodependence of Activity—The thermodependence of activity of Phlig, Eclig, and Tslig is shown in Fig. 3A. It can be seen that the maximal activity of Phlig (16 °C) is shifted toward low temperatures (42 °C for Eclig), reflecting its weak stability. Denaturation of the DNA substrate at high temperatures did not allow the determination of the optimal temperature for Tslig activity, but our results indicate that this value is higher than 55 °C. This highlights a clear link between optimal activity acquisition and thermal adaptation. It should be noted in Fig. 3A that Phlig exhibits significantly higher reaction rates (kcat) at low and moderate temperatures as compared with Eclig and Tslig. Comparison of the temperature dependence of activity (Fig. 3A) and thermal unfolding recorded by fluorescence emission (Fig. 3B) also reveals an interesting behavior of Phlig. Indeed, the maximal activity of Eclig closely corresponds to the beginning of the unfolding transition, therefore explaining the loss of activity at higher temperatures. By contrast, the maximal activity of Phlig is reached 10 °C before unfolding and the enzyme is inactivated at the beginning of the transition. This suggests an extremely labile active site or catalytic intermediates in the cold-adapted enzyme.

Fluorescence Quenching—The conformational flexibility of the three DNA ligases was probed by dynamic fluorescence quenching, using acrylamide as quencher. Fig. 4 shows the Stern-Volmer plots for Phlig (Fig. 4A), Eclig (Fig. 4B), and Tslig (Fig. 4C) at 10 and 25 °C and clearly demonstrates a higher accessibility of tryptophan (W) residues in Phlig as the temperature increases. Absolute values of the Stern-Volmer quenching constant (Ksv) can be compared only if the number and location of W residues in the structures are identical, as well as their environment. This condition is not met for the three related DNA ligases and thus, the variation of the Ksv with temperature becomes the relevant parameter. Indeed, as shown in Fig. 1B, Phlig contains 3 tryptophan residues; Trp-
249 is rather buried, whereas Trp-309 and Trp-583 are relatively accessible to the solvent (Fig. 1).

Finally, the interaction with the oppositely charged DNA substrate, expected to be high temperatures. In the latter, the increase of positively charged amino acids within the active site is likely to facilitate the adaptation to low temperatures of the psychrophile, indicating that the active site of the cold-enzyme is destabilized by an excess of hydrophobic surfaces and contains a decreased number of charged residues compared with its thermophilic counterpart. These findings are in perfect agreement with the kinetic parameters determined for both enzymes (19). Indeed, $h_{\text{coul}}$ is believed to be the most important adaptive parameter for $Ph$lig hence compensating for the effect of low temperatures on the catalytic rate, while for $Ts$lig, the adaptive parameter is expected to be $K_m$ for nicked DNA, which is prone to melting at high temperatures. In the latter, the increase of positively charged amino acids within the active site is likely to facilitate the interaction with the oppositely charged DNA substrate.

### DISCUSSION

**Protein Structure and Active Site Destabilization**—The three-dimensional modeling of the three extremophilic DNA ligases $Ph$lig, $Ec$lig, and $Ts$lig extend previous observations made for the N-terminal domain of $Ph$lig (19) and provide further evidence for the structural determinants implicated in the adaptation to low temperatures of $Ph$lig. Our results suggest that the active site of the cold-enzyme is destabilized by an excess of hydrophobic surfaces and contains a decreased number of charged residues compared with its thermophilic counterpart. These findings are in perfect agreement with the kinetic parameters determined for both enzymes (19).

### Table IV

<table>
<thead>
<tr>
<th></th>
<th>$E_a$ (kJ mol$^{-1}$)</th>
<th>$\Delta H^o$ (kJ mol$^{-1}$)</th>
<th>$\Delta S^o$ (kJ mol$^{-1}$ K$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Ph$lig</td>
<td>300.2$^\circ$K</td>
<td>424.7</td>
<td>93.5</td>
</tr>
<tr>
<td>$Ec$lig</td>
<td>314.5$^\circ$K</td>
<td>164.7</td>
<td>98.1</td>
</tr>
</tbody>
</table>

The absence of heat absorption effects on rescanning of de-natured samples indicates that thermal unfolding of the three enzymes studied is irreversible. The deviation from a two-state model was further confirmed by the fact that the $\Delta H_{\text{cal}}/\Delta H_{\text{eff}}$ ratio for the three enzymes exceeds unity (not shown). The irreversible conformational unfolding of DNA ligases is also highlighted by the scan-rate dependence of their apparent $T_{\text{max}}$ (Fig. 6A), revealing that the thermal denaturation of these proteins is kinetically controlled. Analysis of the thermodynamic parameters of activation for the denaturation process (Fig. 6B and Table IV) indicates that, as expected, the denaturation rate is highest for $Ph$lig (higher $k_{\text{coul}}$) and correspondingly, the energy barrier, $\Delta G^o$, is lowest. However, $E_a$, $\Delta H^o$ and $\Delta S^o$ are all highest for the psychrophile, indicating that the lower thermostability of the cold-adapted DNA ligase is due to an unfavorable entropic contribution. The complex unfolding pattern of $Ts$lig (Fig. 5) precludes such analysis of the kinetically driven denaturation.

**Chemical Stability of DNA Ligases**—The stability of the $Ph$lig, $Ec$lig, and $Ts$lig was further investigated by GdmCl-induced unfolding monitored by intrinsic fluorescence. As shown in Fig. 7, these enzymes unfold according to a three-state pathway, involving a stable intermediate between the native and unfolded states. For the three enzymes, unfolding occurs at distinct denaturant concentrations, with a direct relation between the unfolding transition and the above-mentioned thermal stability. Reversibility of unfolding allowed the determination of the thermodynamic parameters (Table V) i.e. $\Delta G(H_2O)$ (conformational stability in the absence of denaturant), $m$ (slope of the transition, representing the unfolding cooperativity), and $C_m$ (concentration of GdmCl at the transition midpoint). Compared with $Ec$lig and $Ts$lig, the psychrophilic $Ph$lig exhibits the lowest $\Delta G(H_2O)$ and $C_m$ values, indicating its weak stability. In addition, the cold-adapted enzyme possesses the highest $m$ value, reflecting the highest unfolding cooperativity during chemical unfolding.
leading to an improved increase of the charged surface in the order psychrophile the accessible surface of the whole molecule also points out an hydrophobic core is well protected from the solvent by the rigid deed, most native globular proteins do not bind ANS since their experimentally demonstrated by ANS-binding experiments. In- dent the psychrophilic enzyme confirmed this finding, it was ex- destabilize the protein structure (19). While the model of the exposure of hydrophobic residues to solvent, which is expected to part to its increased stability. Indeed, ion pairs in solvent- mesophile 3 demonstrates the exposure to solvent of hydrophobic clusters. Ec
zymes, but in return would be responsible for the weak thermal thermostability of these enzymes. The flexibility concept still gives rise to its increased stability. Indeed, ion pairs in solvent-
activity of psychrophilic enzymes, in addition to their in-
dered the low resistance of the protein to denaturing agents and the high propensity of the structure to unfold at moderate temperatures. The decreased stability of psychrophilic enzymes, in addition to their increased low temperature activity, suggests that there is a direct link between activity and stability i.e. maintenance of activity at low temperatures requires the weakening of intramolecular forces which results in reduced stability. The lower stability of psychrophilic enzymes, brought about by a weakening of intramolecular forces contributing to the cohesion of the native protein molecule, may arise either from a general reduction in strength of intramolecular forces (global flexibility), or from weakened interactions in one or a few important regions of the structure (localized flexibility). Investiga- tion of the thermal stability of Phig by DSC reveals that this enzyme possesses a fragile molecular edifice that is uniformly unstable (one deconvolution unit) and stabilized by fewer weak interactions (decreased \( \Delta H_{\text{cal}} \)) than its mesophilic and thermophilic homologues. It is worth mentioning that Phig displays the lowest calorimetric enthalpy \( \Delta H_{\text{cal}} \) as well as the lowest \( T_m \) ever recorded for cold-adapted enzymes, underlining its strong psychrophilic character through a low conformational stability. Such decreased stability is also highlighted by GdmCl-induced unfolding in which Phig is characterized by the lowest \( \Delta G(H_2O) \) and \( C_m \) values.

Further advances in the understanding of the activity/sta-
bilility relationship can be drawn from the analysis of irreversible thermal unfolding of the cold-adapted DNA ligase and its

### Table V

<table>
<thead>
<tr>
<th>Enzyme (^a)</th>
<th>( \Delta G(H_2O) )</th>
<th>( \Sigma \Delta G(H_2O) )</th>
<th>( m )</th>
<th>( \Sigma m )</th>
<th>( C_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phig</td>
<td>8.4 ± 0.6</td>
<td>27.0</td>
<td>27.7 ± 2.3</td>
<td>43.4</td>
<td>0.3</td>
</tr>
<tr>
<td>f_{N4}</td>
<td>18.6 ± 2.9</td>
<td>31.1</td>
<td>15.7 ± 2.2</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>f_{N4}</td>
<td>12.8 ± 1.2</td>
<td>15.5 ± 2.0</td>
<td>26.1</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Tshig</td>
<td>18.3 ± 3.9</td>
<td>10.6 ± 1.9</td>
<td></td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>f_{N4}</td>
<td>18.2 ± 3.4</td>
<td>11.2 ± 1.8</td>
<td>21.4</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>f_{N4}</td>
<td>42.2 ± 4</td>
<td>10.2 ± 1.0</td>
<td></td>
<td>4.2</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) All thermodynamic parameters were determined according to Ref. 26; \( C_m = \Delta G(H_2O)/m \).
mesophilic counterpart. Our results show an increase in free energies of activation $\Delta G^\circ$ from $P$lig to $E$lig, reflecting that the same denaturation rate $k_{\text{denat}}$ is reached at increasing temperatures (see also Table IV). The small differences in both $\Delta G^\circ$ values arise however from large differences in the enthalpic and entropic contributions. Indeed, the low $\Delta G^\circ$ value recorded for $P$lig corresponds to the largest $\Delta H^\circ$ and $T\Delta S^\circ$ contributions, and conversely for $E$lig. Such trend has already been noticed for a few psychrophilic enzymes (17, 18) and thus seems to be a common property of psychrophilic enzymes.

The low kinetic barrier ($\Delta G^\circ$) recorded for the psychrophilic enzyme allows in fact unfolding at a high rate, leading to a symmetrical and relatively narrow denaturation peak in microcalorimetry (Fig. 5), and consequently to high activation energy $E_a$ and high $\Delta H^\circ$. This reflects a high cooperativity of unfolding for the psychrophilic enzyme, that probably originates from the lower number of interactions required to disrupt the active conformation. The large entropic contribution noticed in $P$lig suggests that its transition state is more disordered than that of its mesophilic counterpart, probably due to the fact that at any specific temperature, more interactions are broken into the psychrophile. The entropy loss associated with hydration of nonpolar groups upon denaturation can also contribute significantly to the differences in entropic contribution between $P$lig and $E$lig. Indeed, in the case of the mesophilic enzyme and more particularly in the thermophilic counterpart, an increased proportion of nonpolar groups are exposed upon unfolding, increasing the order of water molecules and therefore decreasing the entropy change relative to $P$lig. All together, our results perfectly fit those obtained for the icefish glutamate dehydrogenase (16), a cold-adapted $\alpha$-amylase (18) and xylanase (17), demonstrating that thermolability of psychrophilic enzymes is enzymatically driven. The contradictory result obtained by Siddiqui et al. (15) with the elongation factor 2 from a psychrotolerant archaea is likely to arise from an unusual broader DSC profile of the cold enzyme (even though associated with a lower $T_m$) than that of the thermophilic homologue.

Concluding Remarks—Until recently, the activity/flexibility/ stability relationship within psychrophilic enzymes was still appraised with caution. However, the results obtained for the three DNA ligases adapted to different thermal habitats clearly establish a link between activity, flexibility, and stability. The cold-adapted DNA ligase is characterized by a high activity at low temperatures, a high flexibility and a low stability especially at the active site. These results are in perfect agreement with those recently obtained from studies performed on extremophilic $\alpha$-amylases (18) and xylanases (17). Therefore, the emerging picture suggests that psychrophilic enzymes are characterized by increased catalytic efficiency attributed to an increase of the flexibility of appropriate parts of the molecular structure, in order to compensate for the lower thermal energy provided by the low temperature habitat. In return, this flexibility would be provided for the weak thermal and chemical stabilities of cold-adapted enzymes.

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