We describe the properties of a hyperthermophilic, barophilic protease from *Methanococcus jannaschii*, an extremely thermophilic deep-sea methanogen. This enzyme is the first protease to be isolated from an organism adapted to a high-pressure–high-temperature environment. The partially purified enzyme has a molecular mass of 29 kDa and a narrow substrate specificity with strong preference for leucine at the P1 site of polypeptide substrates. Enzyme activity increased up to 116°C and was measured when grown at 130°C, one of the highest temperatures reported for the function of any enzyme. In addition, enzyme activity and thermostability increased with pressure: raising the pressure to 500 atm increased the reaction rate at 125°C 3.4-fold and the thermostability 2.7-fold. Spin labeling of the active-site serine revealed that the active-site geometry of the *M. jannaschii* protease is not grossly different from that of several mesophilic proteases; however, the active-site structure may be relatively rigid at moderate temperatures. The barophilic and thermophilic behavior of the enzyme is consistent with the barophilic growth of *M. jannaschii* observed previously (J. F. Miller et al., Appl. Environ. Microbiol. 54:3039–3042, 1988).

Thermophilic organisms offer a natural source of thermostable proteins for mechanistic studies of protein stability. Evolutionary mechanisms for stabilizing proteins can thus be investigated, pointing the way to natural guidelines for improving protein stability. Specific substitutions suggested by the comparison of thermophilic and mesophilic proteins can guide the construction of stable site-directed mutants (19, 21). In some cases, lessons from nature have been applied in the engineering of more stable variants of existing industrial enzymes (4, 12, 33). Thermophilic enzymes have also been used for the construction of chimeric genes coding for hybrid proteins with thermostabilities intermediate between those of the thermophilic and mesophilic parent proteins (34, 40).

Examining proteins from deep-sea thermophiles affords the additional opportunity of observing how thermostable proteins adapt to a high-pressure environment. This knowledge could become especially useful if applications of high-pressure food processing continue to evolve at a rapid pace (23). Furthermore, increasing evidence that pressure can extend the stable temperature range for a variety of enzymes may encourage the use of biocatalysts in the growing field of high-pressure organic synthesis (18, 43). Particularly notable is a significant number of thermophilic proteins that show improved stability under pressure (28, 29). Studying the properties of proteins adapted to both high pressure and high temperature may further illuminate favorable interactions that allow proteins to remain stable, and microorganisms viable, under a wide range of environmental conditions.

In this report, we describe the properties of a hyperthermophilic, barophilic protease from *Methanococcus jannaschii*, an extremely thermophilic archaean isolated from a deep-sea hydrothermal vent (5, 20). *M. jannaschii* exhibits enhancement of both growth and metabolism by pressure, at high temperatures and pressures extending beyond that of its natural habitat (~250 atm) (31). This enzyme is the first protease to be isolated from an organism adapted to a high-pressure–high-temperature environment.

**MATERIALS AND METHODS**

**Growth of *M. jannaschii***. *M. jannaschii* was grown as described by Tsao et al. (42). Inocula were grown in Hungate bottles and stored anaerobically at 4°C, where they were stable for several months. The medium was as described earlier (42), except that 15 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer was used. Continuously sparged 80% H2 and 20% CO2 substrate gas was initially supplied to the inoculated fermentor at 0.5 liters/min; the flow rate was raised to 1.0 liter/min when growth reached the exponential stage. Cells were harvested from the fermentor when the optical density at 600 nm reached 1.0 by cooling the broth and sparging with N2 to prevent premature cell lysis. Cell pellets were obtained by centrifugation at 10,000 × g for 20 min at 4°C in a Sorvall Superspeed RC-2 centrifuge and stored at −20°C under aerobic conditions.

**Isolation and partial purification of *M. jannaschii* protease**. To obtain crude extracts, the cell pellet was thawed in 10 mM phosphate buffer (pH 7.4) containing 10 mM MgCl2·6H2O and 3 mg of DNase I, and 1% Triton X-100. The mixture (10 ml per g [wet weight] of cells) was agitated for 1 h at 4°C, and the cell debris was removed by centrifugation at 10,000 × g for 30 min at 4°C.

The crude extract was filtered through a 0.22-μm-pore-size membrane (Miltipore) and applied to a DEAE-Sepharose CM-52 column (30 by 8 cm) equilibrated with 50 mM phosphate buffer (pH 7.4) (5 ml/min). The column was washed with 750 ml of the equilibration buffer; proteins, including the protease, were then eluted with 750 ml of a 0 to 300 mM gradient of NaCl in the equilibration buffer. Protease-active fractions were pooled and ultrafiltered at 60 per in2 gauge (psig) over a Millipore YM-10 (size exclusion, 10 kDa) membrane in an Amicon ultrafiltration device (Amicon). The concentrated retentate was then applied to an XAD-7 column (20 by 1.8 cm) equilibrated with 100 mM NaCl in 50 mM phosphate buffer, primarily to remove residual Triton X-100. Nomad-sorbed protein fraction was added to a Sephacryl S-300SF column (115 by 1.8 cm) equilibrated with 50 mM phosphate buffer (pH 7.4) containing 0.1 M NaCl (0.5 ml/min). Active fractions were pooled, reconcentrated, and applied to a second Sephacryl S-300SF column (115 by 1.8 cm) equilibrated with 50 mM phosphate buffer (pH 7.4) containing 1.0 M sodium thiocyanate (NaSCN) (0.3 ml/min). Active fractions were ultrafiltered as described above four times from 20 to 1 ml with 50 mM phosphate buffer (pH 7.4) containing 0.1 M NaCl and 0.05% NaN3 to remove NaSCN. The retentate was used for characterization studies.

**Substrate PAGE**. Discontinuous substrate dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (24) with a 4% stacking gel, except that 0.08% heat-denatured azocasein was incorporated into the 13% separation gel. A 1% stock of azocasein in separation gel buffer was boiled for 20 min prior to addition to the gel. Protein samples and molecular weight markers (Bio-Rad, Hercules, Calif.) were prepared for electrophoresis by incubation with SDS sample buffer (24) for 5 min at 110°C.

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† Present address: EnzyMed, Inc., Iowa City, IA 52242.
Electrophoresis was done at a constant current of 50 mA in a Bio-Rad Mini-PROTEAN II electrophoresis unit.

After electrophoresis, gels were washed twice for 1 h each time with 100 ml of 2.5% Triton X-100 in 50 mM HEPES buffer to replace SDS and separation buffer in the gel, as described by Horie et al. (17). Incubation of the washed gel at 37°C for 30 min in 50 mM HEPES (pH 6.5 at 95°C) produced an azocasein band, and the band was visualized by fixing the gel with ice-cold 15% trichloroacetic acid for 60 min and staining it with Coomassie blue G250 or silver stain.

Protein determination. Protein concentrations were measured by the Bradford method (18).

Table 1. Protease recovery from crude cell extracts of M. jannaschii

<table>
<thead>
<tr>
<th>Lysis technique</th>
<th>Total protein recovered (g)</th>
<th>Total protease activity (U)</th>
<th>Sp act (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis technique</td>
<td>Total protein recovered (g)</td>
<td>Total protease activity (U)</td>
<td>Sp act (U/mg)</td>
</tr>
<tr>
<td>Osmotic shock</td>
<td>6.7</td>
<td>620</td>
<td>0.09</td>
</tr>
<tr>
<td>French press</td>
<td>5.1</td>
<td>710</td>
<td>0.14</td>
</tr>
<tr>
<td>Sonic disruption</td>
<td>2.7</td>
<td>890</td>
<td>0.33</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>8.0</td>
<td>1,600</td>
<td>0.20</td>
</tr>
<tr>
<td>Triton X-100 extraction of cell debris from osmotic shock</td>
<td>1.0</td>
<td>1,030</td>
<td>1.03</td>
</tr>
</tbody>
</table>

* Protease activities were measured in the supernatant as described in the text.

TABLE 1. Protease recovery from crude cell extracts of M. jannaschii

**Results**

**Electrophoresis.** Initial lysis studies on *M. jannaschii* suggested that proteolytic activity is associated with cellular structures; soluble fractions from cells lysed by a French press or osmotic shock yielded relatively low activity after centrifugation, while Triton X-100 extraction or sonication of the cell envelope fraction from such samples yielded substantial activity (Table 1). Negligible protease activity was found in the extracellular fermentation broth.

**Spin labeling and EPR spectroscopy.** Proteases were spin labeled at their active-site serines with 4-(methylfluorophosphinyloxy)-2,2,6,6-tetramethyl-1-piperidinyl. X-p-phenylenediamine, free radical (TEMPO), essentially as described by Morriseit and Broomfield (32). 4-(Methylfluorophosphinyloxy)-TEMPO (150 μl of a 0.1 M solution in isopropanol) was added to 4 ml of protease or partially purified protease solution (2 ml/gm at 4°C in 20 mM phosphate buffer (pH 7.8). The reaction mixture was incubated for 9 h with stirring at 4°C and then slowly warmed to 24°C for further incubation. Residual activity measurements were made periodically to monitor completeness of the labeling reaction. After 48 h, the labeled enzyme was separated from free label by using a Sephadex G-50 size exclusion column, and the protein was concentrated to 10 mg/ml by using an YM-10 ultrafiltration membrane (Amicon). Samples of labeled protease were transferred to glass capillary tubes via a syringe. To obtain the initial spectrum at a given temperature, the sample cavity was first equilibrated at the desired temperature, using a heated nitrogen stream. Electron paramagnetic resonance (EPR) spectra were recorded at the indicated temperature on a Bruker ER200D-SRC spectrometer with a microwave power of 12.6 mW, a modulation amplitude of 2.0 G, and a scan width of 150 G.

**RESULTS**

**Purification and electrophoresis.** Initial lysis studies on *M. jannaschii* suggested that proteolytic activity is associated with cellular structures; soluble fractions from cells lysed by a French press or osmotic shock yielded relatively low activity after centrifugation, while Triton X-100 extraction or sonication of the cell envelope fraction from such samples yielded substantial activity (Table 1). Negligible protease activity was found in the extracellular fermentation broth.

**Purification of *M. jannaschii* protease is summarized in Table 1.** A 580-fold purification with a 29% yield. Substrate (azocasein) SDS-PAGE indicated that this sample (designated 580 PF) contained one band of proteolytic activity at a molecular mass of 29,000 Da (Fig. 1, lane 2). Use of porcine skin gelatin-incorporating gels (17) yielded very tight clearing bands after incubation for 4 h at 95°C, indicating...
only limited hydrolysis of this substrate by *M. jannaschii* protease. A silver-stained, nonsubstrate polyacrylamide gel of the same protease sample indicated that the 580 PF preparation was substantially impure; densitometry indicated that it contained ca. 2% protease among several other proteins (Fig. 1, lane 1).

Gel filtration of the 580 PF fractions on both G-50 and G-100 Sephadex columns (exclusion limit, ~150 kDa) resulted in the elution of a single peak of protein at the void volumes of the columns. Adding 8 M urea to the protease sample and G-100 mobile phase, or heating the protease sample to 95°C immediately before loading on the G-100 column, had no significant effect on protein elution. Under the same conditions, a Sephacryl S-300SF (exclusion limit, 1,500 kDa) calibrated by high-molecular-weight protein standards also yielded a single peak at an apparent molecular mass of 600 to 650 kDa.

Several additional techniques were used in an effort to purify *M. jannaschii* protease further and/or separate it from the multiprotein aggregate. Hydrophobic interaction chromatography on octyl-Sepharose, affinity chromatography on phenyl-Sepharose (36), chromatofocusing, and native PAGE were all ineffective for further purification of active protease. Preparative SDS-PAGE with electroelution or extraction from the gel yielded small quantities of electrophoretically pure protease, but reactivation of the enzyme could be achieved in only very low yields (Table 2).

**pH optimum.** The optimum pH for *M. jannaschii* protease activity toward CBz-AAL-pNA at 95°C was 7.5 to 7.8 (data not shown). Assays of the amidase, esterase, and proteolytic activities of the protease were generally carried out at pH 6.5 instead of 7.8 because of higher background rates and/or turbidity in assay samples at the higher pH.

**Specificity.** The specificity of *M. jannaschii* protease (Table 3) appears to be very narrow, with strong preference for leucine at the P1 site, and considerably less activity toward peptides with shorter aliphatic or aromatic side chains. Secondary specificity for residues beyond the P1 site is indicated by the higher rate for CBz-AAL-pNA than for Ac-Leu-pNA. Esterase activity of the enzyme could be measured only approximately, due to the high background hydrolysis rates at temperatures suitable for activity measurements. In all cases, however, the esterolytic ability of *M. jannaschii* protease was relatively low—less than 20% of the amidase rate on a micromolar basis even for the highly activated *p*-nitrophenyl esters.

**Inhibition.** *M. jannaschii* protease activity could be completely inhibited with 10 mM phenylmethylsulfonyl fluoride and an inhibitor of serine proteases (and sometimes of cysteine protease) (37). Inhibition by phenylmethylsulfonyl fluoride was reversible by incubation with 10 mM dithiothreitol, nor was the enzyme inhibited by iodoacetate, indicating that the *M. jannaschii* protease is not a cysteine protease (37). Furthermore, despite the enzyme’s high amidase activity, it was not inhibited by the aminopeptidase inhibitor bestatin (14, 37). The lack of inhibition by EDTA indicated that the protease contains no specific divalent ion binding sites important to its function.

### TABLE 3. Specificity of *M. jannaschii* protease

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBz-AAL-pNA</td>
<td>100</td>
</tr>
<tr>
<td>Boc-Ala-Ala-Pro-Ala-pNA</td>
<td>10</td>
</tr>
<tr>
<td>Suc-Ala-Ala-Pro-Phe-pNA</td>
<td>5.4</td>
</tr>
<tr>
<td>Ala-Ala-Phe-pNA</td>
<td>4.8</td>
</tr>
<tr>
<td>Ac-Leu-pNA</td>
<td>3</td>
</tr>
<tr>
<td>Bz-Tyr-pNA</td>
<td>7.8</td>
</tr>
<tr>
<td>Bz-Gly-pNA</td>
<td>3.6</td>
</tr>
<tr>
<td>Leu-pNA</td>
<td>15</td>
</tr>
<tr>
<td>Phe-pNA</td>
<td>2.7</td>
</tr>
<tr>
<td>Boc-Leu-Gly-Arg-pNA</td>
<td>ND*</td>
</tr>
<tr>
<td>Ac-Leu-OMe</td>
<td>ND</td>
</tr>
<tr>
<td>CBz-Leu-ONp</td>
<td>5.1</td>
</tr>
<tr>
<td>CBz-Ala-ONp</td>
<td>1.2</td>
</tr>
<tr>
<td>Fua-Leu-OMe</td>
<td>ND</td>
</tr>
<tr>
<td>Ac-Phe-OEt</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, none detected.
Effects of temperature and pressure. The Arrhenius plot for azocasein hydrolysis by the 580 PF protease (Fig. 2A) indicates that proteolytic rates increased up to 120°C. The activity of the M. jannaschii protease increased up to 116°C. This is one of the highest optimum temperatures for a proteolytic enzyme reported in the literature (3, 6, 8, 10, 11, 27, 39, 44). The maximum temperature for which enzyme activity was measured, 130°C, is among the highest measured temperatures reported for the function of any enzyme. Similar results were obtained using cell extracts (data not shown), indicating that the maximum and optimum temperatures were independent of enzyme purity up to a purification factor of at least 580-fold.

The Arrhenius plot (Fig. 2B) of amidase activity between 65 and 130°C is also biphasic, exhibiting a discontinuity in the slope at ~87°C. This corresponds to a change in the activation energy for catalytic activity from $E_a = 53 \text{ kJ/mol}$ below 80°C to $E_a = 14 \text{ kJ/mol}$ above 95°C.

Figure 3 presents data collected for the 580 PF protease over a range of temperatures at three different pressures in the high-pressure reactor. The theoretical curve fits were generated by fitting the high-pressure data to the temperature-dependent rate equation described by Hei and Clark (16). As shown, pressure substantially enhances the activity of the protease at each temperature. Raising the pressure to 500 atm increased the maximum reaction rate twofold and the rate at 130°C fivefold.

Stability. The thermal half-life of the protease is exceptionally high, for example, 45 min at 116°C and 7 min at 125°C (at ~10 atm of pressure). The protease from M. jannaschii is therefore one of the most thermostable proteins ever characterized. By comparison, the reported half-life of pyrolysin, a hyperthermophilic protease from Pyrococcus furiosus, is 3 min at 110°C (11). A more complete comparison of protease half-lives is shown in Fig. 4, which plots half-lives versus temperature for several thermophilic proteases. Moreover, the thermostability of M. jannaschii protease increased with pressure in a physiologically relevant range. Pressure effects on protease stability are summarized in Table 4. For example, raising the pressure to 500 atm increased the half-life 2.7-fold at 125°C. Consistent with the activity studies, the thermostability and the degree of pressure stabilization were independent of protease purity (data not shown).

FIG. 2. (A) Arrhenius plot for proteolytic activity of M. jannaschii protease. Values represent averages of triplicate experiments. (B) Arrhenius plot for amidase activity of M. jannaschii protease. Values are averages of four measurements. T, temperature.

The spectrophotometric initial-rate assay with the synthetic peptide derivative CBz-AAL-pNA allowed rapid assays with relatively low (~10%) background hydrolysis up to 130°C. The activity of the M. jannaschii protease increased up to 116°C. This is one of the highest optimum temperatures for a proteolytic enzyme reported in the literature (3, 6, 8, 10, 11, 27, 39, 44). The maximum temperature for which enzyme activity was measured, 130°C, is among the highest measured temperatures reported for the function of any enzyme. Similar results were obtained using cell extracts (data not shown), indicating that the maximum and optimum temperatures were independent of enzyme purity up to a purification factor of at least 580-fold.

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Spin-labeling studies. The spin label 4-(methylfluorophosphinyloxy)-TEMPO is a highly selective suicide inhibitor of serine proteases, reacting exclusively with the active-site serine (32). Therefore it was possible to perform spin-labeling studies on the impure sample of *M. jannaschii* protease. The EPR spectra of four serine proteases, chymotrypsin, trypsin, subtilisin Carlsberg, and the 580 PF *M. jannaschii* protease, are shown in Fig. 5. All four spectra were recorded at 20°C. The spectral line shapes are very similar, indicating that the active-site geometry of *M. jannaschii* protease is not grossly different from that of its mesophilic counterparts, at least at 20°C. On the other hand, Fig. 6 compares the EPR spectra of spin-labeled trypsin and *M. jannaschii* protease at 2 and 60°C. Over this temperature range, the spectrum of trypsin changes significantly, reflecting increased conformational fluctuation and eventual thermal denaturation of the protein as the temperature is raised. In contrast, the spectrum of the *M. jannaschii* protease remains unchanged, indicating that the active-site structure is insensitive to temperature from 2 to 60°C. Unfortunately, release of the spin label at temperatures above 60°C precluded spectroscopic examination of the *M. jannaschii* protease at higher temperatures.

**DISCUSSION**

*M. jannaschii* protease is an intracellular, apparently membrane-associated protease that appears to function as a multimeric aggregate. The tenacity of the aggregate form, which is resistant to separation by a number of techniques under a wide range of conditions (chaotropic agents, temperature, salts, and some surfactants), suggests that the aggregate is the likely physiological form of the protease in vivo. Similar strongly associated protein complexes have been observed for several other extremely thermophilic enzymes. For example, protein

![FIG. 4. Thermal stabilities of thermophilic proteases. Data sources: ●, this study; ▲, reference 11; □, reference 10; ■, reference 6; △, reference 8; ○, reference 39. T<sub>opt</sub>, optimal temperature; S., Sulfolobus.](Image)

![FIG. 5. EPR spectra of spin-labeled proteases at 20°C.](Image)

![FIG. 6. EPR spectra of spin-labeled trypsin and *M. jannaschii* protease at 2 and 60°C.](Image)

![TABLE 4. Stabilization of *M. jannaschii* protease by pressure](Image)

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th><em>t</em>&lt;sub&gt;1/2&lt;/sub&gt; (min)</th>
<th><em>t</em>&lt;sub&gt;1/2&lt;/sub&gt; (500 atm)/<em>t</em>&lt;sub&gt;1/2&lt;/sub&gt; (10 atm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>110</td>
<td>170</td>
<td>320</td>
</tr>
<tr>
<td>116</td>
<td>45</td>
<td>83</td>
</tr>
<tr>
<td>120</td>
<td>18</td>
<td>45</td>
</tr>
<tr>
<td>125</td>
<td>7</td>
<td>19</td>
</tr>
</tbody>
</table>

*<sup>a</sup>*<sub>*t*<sub>1/2</sub>, half-life.
aggregation has hindered efforts to purify thermophilic aqua-
lysin II from *Thermus aquaticus* YT-1 (26), a carboxylesterase from the thermophilic Bacillus strain G18A7 (35), and a protease from *Thermus caldophilus* GK-24 (41). Kelly et al. (22) speculate that the apparent propensity of many thermophilic proteins to aggregate may be related to a high percentage of hydrophobic amino acids.

*M. jannaschii* protease was expressed at very low levels (ca. 1 to 5 µg/liter of fermentation volume), suggesting that the enzyme serves a specific physiological role in the cell (such as posttranslational processing of an apoprotein), rather than a general nutritional or protein turnover role. The complete genome of *M. jannaschii* has recently been reported (5). Comparing the primary sequences of genes putatively identified to encode proteolytic enzymes with the molecular mass of the protease (determined by substrate gel electrophoresis) provides an approximate match with the proteasome α subunit. However, less than 50% of *M. jannaschii* genes could be assigned, and so it is possible that the present protease is coded for by an unrecognized gene. Thus, it is not presently possible to correlate any of the identified genes with the *M. jannaschii* protease.

The turnover number for this protease could not be calculated directly since it was not completely purified, nor could the active-site concentration be determined. However, based on densitometry of the silver-stained 580 PF protease and the molecular weight of the active band, *k* cat for amidase activity was estimated to be 3,000 s⁻¹ at the optimum temperature of 116°C. By comparison, common mesophilic digestive proteases (e.g., subtilisin, chymotrypsin, and trypsin), typically have maximum turnover numbers of about a few hundred per second for synthetic peptide p-nitroanilides at their optimum temperatures. In addition, comparison of the specific amidase activity with data for several other thermophilic proteases described in the literature (9, 14) suggests that the 580 PF protease is unusually active.

The high-pressure experiments suggest mechanisms by which the protease maintains its catalytic integrity at such high temperatures. Stabilization of a protein to irreversible inactivation can occur by stabilizing the native structure of the protein against reversible unfolding and/or by inhibiting irreversible degradation reactions. The predominant reactions known to affect irreversible protein inactivation (i.e., deamination, hydrolysis of peptide bonds, breakage of disulfides and destruction of Cys, amino acid racemization, and lysinoalanine formation) are believed to proceed through polar transition states (29). The increased polarity leads to a reduction in the partial molar volume of the solvent due to the phenomenon of electrostriction (29, 43); therefore, such a process is favored by increased pressure. High pressure would thus be expected to accelerate protein inactivation unless the native conformation is stabilized by pressure, which appears to be the case for the *M. jannaschii* protease.

Of the primary intramolecular interactions responsible for maintaining native protein structure, only hydrophobic interactions are expected to be stabilized by elevated pressure. This effect has been attributed to the relatively open structure of water solvating apolar surfaces exposed during protein unfolding (16, 29). In contrast, intraprotein electrostatic interactions (salt bridges or ion pairs) are strongly destabilized by increasing pressure (29). But intramolecular hydrogen bonds readily exchange with solvating water molecules upon protein unfolding. Hydrogen bonds are therefore expected to have little net influence on the stability of proteins under pressure. Thus, proteins stabilized primarily by hydrophobic interactions would be expected to be stabilized by high pressure, whereas proteins stabilized by significant electrostatic interactions should be destabilized by pressure (i.e., the disruption of electrostatic interactions that accompanies protein unfolding will lead to increased electrostriction and a reduction in volume). This reasoning, together with the aggregation behavior of the protease, suggests that the *M. jannaschii* protease relies to a large extent on hydrophobic interactions for its exceptional thermostability. Pressure-stability studies may therefore provide a rapid and general, although indirect, way of examining the primary mechanisms of protein thermostability. For homologous proteins from phylogenetically related sources, such analysis could offer unique insights into evolutionary mechanisms for regulating thermostability.

The spin-labeling results suggest that the *M. jannaschii* protease has limited conformational flexibility at temperatures below ~60°C. A relatively rigid structure may contribute to the low catalytic activity of the enzyme at ambient temperature. Previous studies of a thermophilic lactate dehydrogenase and a halophilic dehydrogenase support the notion that the specific activity and thermal stability of enzymes are regulated by the flexibility of the polypeptide chain (15). Thus, in addition to their practical advantages, thermophilic enzymes may provide a convenient means to examine the role of protein flexibility and dynamics in enzyme activity and stability at moderate temperatures.

In general, the present results support the hypothesis that thermophilic proteins become increasingly rigid (and inactive) at temperatures below their normal physiological function. The same structural features and physical interactions that confer protein stability at high temperatures may therefore preclude sufficient flexibility for catalysis at lower temperatures. On the other hand, mesophilic proteins, which may have evolved from thermophilic ancestors, may have reduced their thermostability in order to obtain sufficient flexibility for catalytic activity at lower temperatures.

*M. jannaschii* protease activity was enhanced 400% at 500 atm and 130°C, corresponding to a activation volume (ΔV*) of ~106 cm³/mol. This value is substantially higher than those typically observed for terrestrial, mesophilic serine proteases. For example, the activation volumes for several serine proteases reported in the literature range from +27 to ~33 cm³/mol (28). The most negative activation volume previously reported, ~33 cm³/mol, was for the hydrolysis of Suc-Ala-Ala-pNA by α-chymotrypsin (25). The effect of pressure on *M. jannaschii* protease stability also appears to be unusual: when 500 atm was applied to trypsin, α-chymotrypsin, and subtilisin Carlsberg, half-lives at the enzyme’s reported melting temperature increased by 40, 30, and 30%, respectively (data not shown), compared to 170% for *M. jannaschii* protease at 125°C.

The barophilic behavior of the *M. jannaschii* protease is especially noteworthy in view of the unusual barophily exhibited by *M. jannaschii* in previous studies (31). Moreover, this protease is not the only enzyme from *M. jannaschii* whose activity is enhanced by pressure; the hydrogenase activity in crude extracts of *M. jannaschii* more than tripled when the pressure was raised from ca. 7.5 to 260 bars at 86°C (30). Both enzymes were also substantially stabilized by pressure at high temperatures. Whether barophilic growth of *M. jannaschii* can be directly attributed to pressure activation of key enzymes remains to be seen; however, the similar effects of pressure on growth and enzyme activity suggest that these phenomena are interrelated and motivate further pressure studies of enzymes from *M. jannaschii* and other deep-sea thermophiles.
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