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Pressure enhances thermal stability of DNA polymerase from three thermophilic organisms

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Abstract DNA polymerases derived from three thermophilic microorganisms, *Pyrococcus* strain ES4, *Pyrococcus furiosus*, and *Thermus aquaticus*, were stabilized *in vitro* by hydrostatic pressure at denaturing temperatures of 111°C, 107.5°C, and 100°C (respectively). Inactivation rates, as determined by enzyme activity measurements, were measured at 3, 45, and 89 MPa. Half-lives of *P.* strain ES4, *P. furiosus*, and *T. aquaticus* DNA polymerases increased from 5.0, 6.9, and 5.2 minutes (respectively) at 3 MPa to 12, 36, and 13 minutes (respectively) at 45 MPa. A pressure of 89 MPa further increased the half-lives of *P.* strain ES4 and *T. aquaticus* DNA polymerases to 26 and 39 minutes, while the half-life of *P. furiosus* DNA polymerase did not increase significantly from that at 45 MPa. The decay constant for *P.* strain ES4 and *T. aquaticus* polymerases decreased exponentially with increasing pressure, reflecting an observed change in volume for enzyme inactivation of 61 and 73 cm^3/mol, respectively. Stabilization by pressure may result from pressure effects on thermal unfolding or pressure retardation of unimolecular inactivation of the unfolded state. Regardless of the mechanism, pressure stabilization of proteins could explain the previously observed extension of the maximum temperature for survival of *P.* strain ES4 and increase the survival of thermophiles in thermally variable deep-sea environments such as hydrothermal vents.

Key words Hydrostatic pressure · Hyperthermophile · DNA-dependent DNA polymerase · Protein stability · Thermostability

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Introduction

The interaction of temperature and pressure on microbial growth can provide new insights into microbial physiology and biochemistry. While it is not uncommon for deep-sea isolates to be barophilic, demonstrating their maximum growth rates at their habitat pressure (Yayanos 1995), some deep-sea hyperthermophiles exhibit unusual responses to pressure at increased temperatures. *Pyrococcus* strain ES4 and *Thermococcus* strain ES1 are barophilic primarily at temperatures at or above their optimal temperature at atmospheric pressure (Pledger et al. 1994; Nelson et al. 1992), and *P.* strain ES4 and *P. abyssi* experience an upward shift in their optimum temperature for growth under 20–40 MPa pressure (Pledger et al. 1994; Erauso et al. 1993). The most widespread effect seen with hyperthermophiles is a 1°–3°C extension of the maximal growth temperature under deep-sea pressures: examples include *Desulfurococcus* strain SY and *P.* strain GB-D (Jannasch et al. 1992), *P. abyssi* (Erauso et al. 1993), *T. peptonophilus* (Canganella et al. 1997), and *T.* strain ES1 and *P.* strain ES4 (Pledger et al. 1994).

P. strain ES4 (Pledger and Baross 1991), a hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent, increased its maximum growth temperature by 2°C under a presumably native 22-MPa pressure (Pledger et al. 1994). The heat-shock temperature in ES4 was also raised approximately 2°C under pressure, as indicated by increased thermotolerance and the accumulation of a 98-kDa heat-stress protein marker (Holden and Baross 1995). The delay in the onset of heat-shock might be explained by pressure stabilization of proteins from ES4, as it has been shown previously that relatively low, ecologically relevant pressures (10–50 MPa) can stabilize proteins under thermal stress (e.g., Johnson and Campbell 1946), including proteins from hyperthermophiles (Michels et al. 1996). We sought to determine whether pressure-induced protein thermostabilization occurs in ES4.

The stability of DNA polymerase from *P.* strain ES4 was studied under conditions of elevated temperature and pres-

sure to determine whether pressure increases the thermal stability of this enzyme. Though ES4 DNA polymerase is remarkably thermostable compared to most known enzymes, DNA polymerases are the most heat-labile enzymes known from *Pyrococcus* species (Adams 1992). The DNA polymerase was selected as an ecologically relevant indicator of physical stabilization effects because of its relatively low stability and importance in cell reproduction.

Most documented temperature-related pressure responses are in deep-sea hyperthermophilic isolates. In order to determine whether protein thermostabilization by pressure is restricted to species from the deep ocean, we also studied the pressure response of two other DNA polymerases. Both *P. furiosus* (Fiala and Stetter 1986), a hyperthermophilic species isolated from shallow marine hot springs and closely related to *P.* strain ES4, and *Thermus aquaticus* (Brock and Freeze 1969), a moderately thermophilic bacterium isolated from terrestrial hot springs, produce commercially important DNA polymerases which were tested for pressure-enhanced thermostability. DNA polymerases from *P.* strain ES4, *P. furiosus*, and *T. aquaticus* were thermally stressed using the "winerack" pressure system (Pledger et al. 1994) and the remaining enzyme activity was followed through time to determine inactivation rates at denaturing temperatures and increasing pressures.

Methods

DNA polymerase stocks. Purified recombinant DNA polymerases from *P. furiosus* (Pfu polymerase, Lundberg et al. 1991) and *T. aquaticus* (Taq polymerase, Chien at al. 1976) as well as partially purified cloned DNA polymerase from *P.* strain ES4 were supplied by Stratagene (La Jolla, CA, USA). The enzymes were diluted to approximately 50 units/ml (equivalent to 1–10 μg/ml) in Tris-based assay buffer to form a working stock stored at 4°C. Tris buffers are relatively insensitive to pressure changes (Neuman et al. 1973). Buffers for *T. aquaticus* and *P. furiosus* DNA polymerases were obtained from Stratagene; *P.* strain ES4 DNA polymerase performs best in Vent buffer from New England BioLabs (Beverly, MA, USA). Salt concentrations in these buffers are 10 mM KCl, 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, and 2 mM $MgSO_4$.

Pressure experiments. Pressure studies were carried out in the "winerack" system (Pledger et al. 1994), utilizing one stainless steel pressure vessel (TemPress, Leco, State College, PA, USA) and computer-controlled oven. DNA polymerase solutions were placed in three to six 1-ml syringes, loaded into the pressure cylinder, and pressurized using water as the hydraulic fluid. Temperature was continuously monitored via a thermocouple inserted into the back of the cylinder; it took approximately 40 min for the vessel to reach the stress temperature. Once the target temperature was reached, the cylinder was allowed to incubate for a measured time, during which the temperature and pressure were maintained at ±0.3°C and ±1 MPa, respectively. At the end of the incubation, the pressure vessel was immediately chilled in a room-temperature water bath; samples reached room temperature 2–4 min after the cylinder was removed from the oven. Stressed enzyme solutions were stored at 4°C for up to 2 weeks before they were assayed for remaining activity. Enzyme solutions did not change in activity over this time period.

DNA polymerase activity assay. DNA polymerase activity was measured using a modification of a calf thymus DNA gap-filling assay (Grippo and Richardson 1971; Maki et al. 1988). The assay cocktail contained 0.25 mg/ml gapped calf thymus DNA (Pharmacia Biotech Piscataway, NJ, USA), 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/ml nuclease-free bovine serum albumin (BSA) (Stratagene), 0.03 mM dATP, 0.03 mM dCTP, 0.03 mM dGTP, 0.003 mM dTTP, and 50 μCi/ml [^{3}H]TTP (20 Ci/mmol in 50% ethanol, lyophilized before use, Dupont NEN, Boston, MA, USA). Temperature-stressed DNA polymerase solutions and assay cocktail were mixed in equal proportions, incubated at 72°C for 30 min, and spotted onto Whatman DE81 filters (Whatman, Clifton, NJ, USA). The filters were dried completely and unincorporated tritiated thymidine triphosphate was rinsed off with standard saline citrate (0.15 M NaCl, 15 mM sodium citrate, pH 7.0; 4 × 5 minute rinses). The filters were dried again after a 30-s rinse in ice-cold 95% ethanol. Each filter was counted in a Minaxi TriCarb 4000 series (Packard) scintillation counter to measure the amount of tritiated thymidine successfully incorporated into the gapped DNA, providing an estimate of the concentration of active DNA polymerase in the temperature-stressed solution.

Enzyme inactivation profiles. Enzyme inactivation profiles were generated from replicate experiments that were allowed to incubate at high temperature for various lengths of time. In some cases, as much as 60% of the DNA polymerase activity was lost during initial heating of the pressure cylinder, so all measurements were normalized to the activity remaining at the start of incubation and are expressed as percent active enzyme. Activity data were regressed against time to determine enzyme half-lives, which were compared using an ANCOVA test followed by a Tukey multiple comparisons test (Zar 1984).

Results

Selection of experimental temperatures. Preliminary studies were undertaken to determine the optimal temperatures at which to stress DNA polymerases from *P.* strain ES4, *P. furiosus*, and *T. aquaticus.* The stress temperatures needed to be high enough to produce a measurable decrease in the amount of active enzyme within 2 h (due to equipment limitations) and low enough to preserve enough activity to calculate a half-life with confidence. The stability of the three DNA polymerases studied was sensitive to temperature at

low pressure (for example, *P.* strain ES4 DNA polymerase as shown in Fig. 1). For simplicity, we compared the pressure response of the three enzymes at temperatures that produced equivalent low-pressure (3-MPa) half-lives. Based on these initial results, we chose stress temperatures for each enzyme that produced nearly a 5-min half-life at 3 MPa: 111°C for *P.* strain ES4 DNA polymerase, 107.5°C for *P. furiosus* DNA polymerase, and 100°C for *T. aquaticus* DNA polymerase. During the initial experiments, we also confirmed that the native pressure of *P.* strain ES4, 22 MPa, partially stabilized its DNA polymerase (data not shown).

Pressure stabilization of DNA polymerases. Pressure enhanced the thermostability of all three enzymes (Fig. 2). The enzyme decay profiles were best fit by exponential decay, indicating first-order kinetics. For each enzyme, the half-life at 45 MPa was significantly longer ($P < 0.001$) than the half-life at 3 MPa (Table 1). *P.* strain ES4 and *T. aquaticus* DNA polymerases showed further stabilization with 89 MPa pressure ($P < 0.001$), while the half-life of *P. furiosus* DNA polymerase did not increase significantly beyond 45 MPa.

Comparison of polymerases. The ratio of the half-life at moderate pressure to that at low pressure gives a measure of the pressure stabilization experienced by the enzyme (Table 1). *P.* strain ES4 and *T. aquaticus* polymerases showed equivalent degrees of stabilization at each of the higher pressures ($P > 0.5$). The stabilization of *P. furiosus* DNA polymerase at 45 and 89 MPa was not significantly different from that of *P.* strain ES4 and *T. aquaticus* DNA polymerase at 89 MPa ($P > 0.2$).

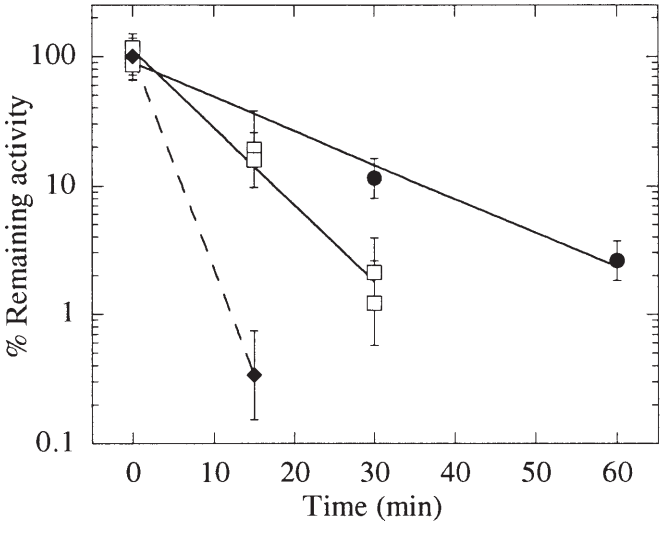

Fig. 1. Remaining activity of *Pyrococcus* strain ES4 DNA polymerase at 3 MPa and 110°C (*filled circle*), 111°C (*open square*), and 112.5°C (filled diamond). *Error bars* denote 95% confidence intervals. First-order decay curves are shown, corresponding to half-lives of 10, 5, and 1.8 min, respectively. The DNA polymerase activity after incubation at 112.5°C was at the detection limit, so any calculated half-life will be an upper limit

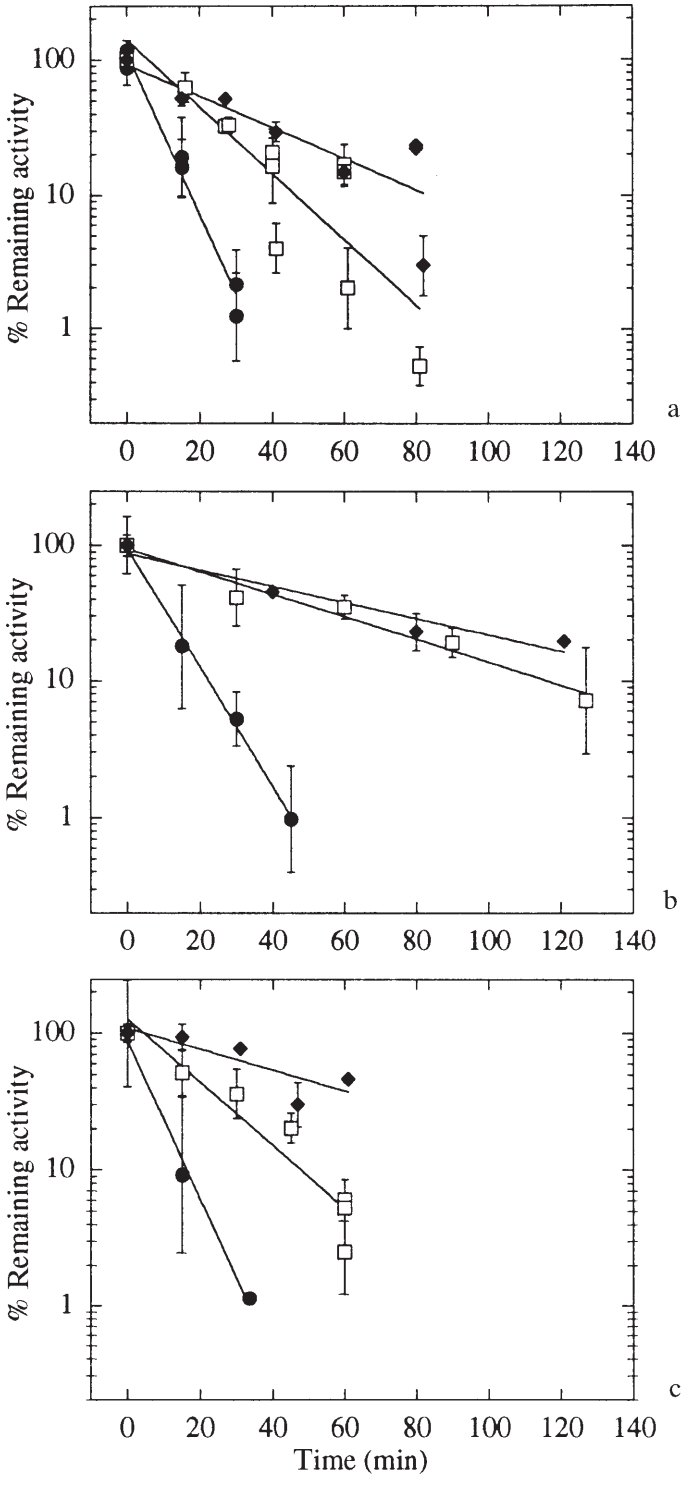

Fig. 2. Remaining activity of **a** *Pyrococcus* strain ES4 DNA polymerase at 111°C, **b** *Pyrococcus furiosus* DNA polymerase at 107.5°C, and **c** *Thermus aquaticus* DNA polymerase at 100°C vs time at 3 MPa (*filled circle*), 45 MPa (*open square*), and 89 MPa (*filled diamond*). *Error bars* denote 95% confidence intervals but were not plotted if smaller than the symbol. First-order decay curves are shown

Table 1. Pressure effects on the thermostable DNA polymerases

Organism	Source	Growth optimum	Stress temperature	Half-lives[a] (min) at			Ratio of half-lives[a]	
				3 MPa	45 MPa	89 MPa	45/3 MPa	89/3 MPa
Pyrococcus strain ES4	Deep marine (>2000 m)	95°C	111°C	5.0 (4.5, 5.7)	12 (10, 15)	26 (21, 35)	2.4 (1.8, 3.3)	5.2 (3.7, 7.8)
Pyrococcus furiosus	Shallow marine	95°C	107.5°C	6.9 (5.8, 8.3)	36 (30, 45)	50 (41, 62)	5.2 (3.7, 7.8)	7.2 (4.9, 11)
Thermus aquaticus	Terrestrial freshwater	70°C	100°C	5.2 (4.2, 6.8)	13 (11, 15)	39 (29, 63)	2.5 (1.6, 3.6)	7.5 (4.3, 15)

[a] 95% confidence intervals for half-lives are asymmetric and shown in parentheses.

Discussion

Pressure stabilization of proteins at stressful temperatures has previously been demonstrated with such varied proteins as γ-globulin and ovalbumin (Suzuki and Taniguchi 1972), pyrophosphatase (Morita and Mathemeier 1964), and hydrogenase (Hei and Clark 1994). The current study is the first to test this effect with a DNA polymerase, and, moreover, from an organism that increases its maximum growth temperature with the addition of pressure (*P.* strain ES4). The DNA polymerase from the deep-sea hyperthermophile *P.* strain ES4 was stabilized by moderate pressure, as were the DNA polymerases from *P. furiosus* and *T. aquaticus*, thermophilic organisms cultured from low-pressure environments. The similar response to pressure of the three DNA polymerases in this study shows that pressure stabilization is not unique to enzymes from deep-sea organisms.

An analogous enzyme stability study by Hei and Clark (1994) also found that pressure stabilization of enzymes was unrestricted to organisms from the deep sea. Instead, the pressure responses of hydrogenase, α-glucosidase, and glyceraldehyde-3-phosphate dehydrogenase were correlated with the optimum growth temperature of the organisms from which the proteins were purified. This relationship led to the conclusion that pressure stabilization is more likely (and perhaps common) for thermophilic enzymes than for mesophilic enzymes. The DNA polymerase data presented here are consistent with these findings; no DNA polymerases from mesophilic organisms have been tested to date. Though their original conclusion regarding preferential pressure stabilization of proteins from thermophiles has not proven to be fully general [rubredoxin (Hei and Clark 1994) and adenylate kinase (Konisky et al. 1995) from thermophiles are destabilized by pressure], it may yet reflect common structural characteristics of proteins that permit favorable interactions with pressure.

In this study, we measured DNA polymerase stability at three pressures and were able to show that, for the moderate pressures tested, increasing pressure increased stability. That relationship indicates that the protein destabilization reaction involves a change in volume, and furthermore, that the change in volume is positive. As pressure increases at constant temperature, reactants will be favored over products if the system volume is larger after the reaction is complete. Expressed using classical thermodynamics, that statement becomes

$$\left(\partial \ln K_{eq}/\partial P\right)_T = -\Delta V/RT \qquad \text{(Eq. 1)}$$

where R is the universal gas constant, T is the absolute temperature, K_{eq} is the equilibrium constant for a reaction, and ΔV is the difference in volume between the products and reactants. A similar relationship governs the rate constant for a reaction (k)

$$\left(\partial \ln k/\partial P\right)_T = -\Delta V^{\ddagger}/RT \qquad \text{(Eq. 2)}$$

where $\Delta V^{\ddagger}$ is the volume of activation, the difference in volume between the reactants and the transition state. Effects of pressure on equilibria and reaction rates are detailed in reviews by Johnson and Eyring (1970), Morild (1981), and Michels and Clark (1992). For the inactivation of *P.* strain ES4 and *T. aquaticus* DNA polymerases, we were able to fit the observed enzyme inactivation rates (k_{obs}) to the general relation

$$\left(\partial \ln k_{obs}/\partial P\right)_T = -\Delta V_{obs}/RT \qquad \text{(Eq. 3)}$$

to obtain an observed change in volume (ΔV_{obs}) of 61 and 73 cm^3/mol, respectively (Fig. 3). This stabilization is close to that seen by Hei and Clark (1994) for the thermostable hydrogenases from *Methanococcus jannaschii* and *M. igneus* (95 and 91 cm^3/mol, respectively) and for *P. furiosus* α-glucosidase (60 cm^3/mol) and may reflect similar means of pressure stabilization.

The observed change in volume must be dissected into its fundamental volumes before a physical interpretation can be made. The canonical mechanism for enzyme inactivation

$$\text{Native} \xleftrightarrow{K_{rev}} \text{Denatured} \xrightarrow{k_{irrev}} \text{Inactive}$$

holds that the native, active enzyme reversibly unfolds to reach equilibrium (K_{rev} = [D]/[N]) with a denatured state which decays at a fixed rate (k_{irrev}) to a irreversibly inactivated form (Lumry and Eyring 1954; Zale and Klibanov 1983). The observed rate constant may be expressed in terms of the equilibrium constant for unfolding and the rate constant for inactivation (Zale and Klibanov 1983):

$$k_{obs} = k_{irrev}K_{rev}/\left(1 + K_{rev}\right) \qquad \text{(Eq. 4)}$$

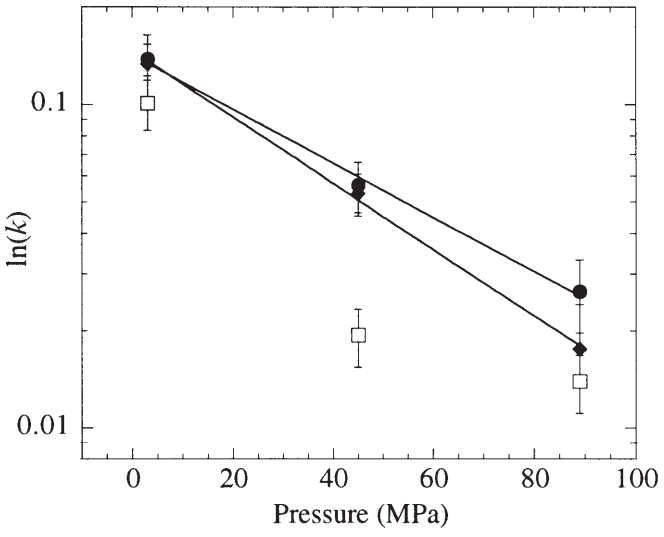

Fig. 3. Observed enzyme activity decay constants (k_{obs}) for DNA polymerases from *Pyrococcus* strain ES4 (*filled circle*), *Pyrococcus furiosus* (*open square*), and *Thermus aquaticus* (*filled diamond*) vs pressure. *Error bars* denote 95% confidence intervals. The rate constants for *P.* strain ES4 and *T. aquaticus* DNA polymerases show variation with pressure consistent with the theoretical exponential relationship (Eq. 3). ΔV for *P.* strain ES4 DNA polymerase is $+61\,cm^3/mol$ and ΔV for *T. aquaticus* DNA polymerase is $+73\,cm^3/mol$

each of which may be affected by pressure. Enzyme inactivation studies cannot easily separate the two; we can, however, narrow the possibilities for pressure influence in both categories. Some plausible explanations for the observed positive change in volume for the inactivation of thermostable enzymes are presented here.

Inactivation of the protein may proceed through a step that increases the system volume ($\Delta V^{\ddagger}_{irrev} > 0$). Common mechanisms of protein inactivation include those that involve the entire protein, such as aggregation or general conformational change, and those that involve localized portions of the protein, including deamidation, peptide backbone hydrolysis, and disulfide link destruction (Volkin and Klibanov 1992). Mechanisms that involve only portions of the protein are unlikely to account for a large transient increase in volume ($60–90\,cm^3/mol$). Aggregation can involve most of the protein, and as pressure has been shown to dissociate multimeric proteins (Silva and Weber 1993), increased pressure might also retard aggregation of proteins. However, the observed first-order kinetics of inactivation exhibited by the DNA polymerase imply that a single protein molecule is involved in inactivation and therefore that aggregation cannot be a dominant method of inactivation in this case. A unimolecular inactivation mechanism is responsible for the demise of these DNA polymerases, and if the inactivation reaction is the basis for the pressure stabilization then the inactivation reaction is likely to involve much of the protein.

Reversible protein unfolding may also increase the volume of the system ($\Delta V_{rev} > 0$). The largest change in volume in reversible protein unfolding will be linked to solvation of amino acid residues (Michels et al. 1996), especially the transfer of buried residues into the solvent. The change in volume for solvation of hydrophobic residues may be positive (Hvidt 1975), though the community is still unsure (Kauzmann 1987; Michels et al. 1996). Proteins with extremely hydrophobic centers would then be likely to increase the overall volume of the system when unfolded (Hei and Clark 1994). The DNA polymerases in this study are quite hydrophobic overall, as the calculated bulk transfer free energy densities of *P. furiosus* and *T. aquaticus* DNA polymerases are 40.7 and 41.6 J/g, respectively, calculated by the method of Dale and McBennett (1992) using sequences from GenBank. [For reference, Dale and McBennett (1992) have calculated bulk transfer free energy densities for 10 proteins and values range from 26.8 J/g for bovine pancreatic ribonuclease to 39.1 J/g for sperm whale metmyoglobin.] The three thermostable DNA polymerases studied might be stabilized due to increased hydrophobic interactions.

Rather than being specific to the structure of thermostable proteins, protein stabilization by pressure at high temperatures might reflect a more general feature of protein unfolding in water, perhaps independent of changes in primary structure. The change in volume for protein unfolding seems to increase with increasing temperature (Michels et al. 1996), and Morild (1981) notes that the change in volume for protein unfolding can change sign from negative to positive in the temperature interval between 20°C and 100°C. These effects are seen in a paper by Taniguchi et al. (1994) where chymotrypsinogen and ribonuclease A stabilities are plotted in temperature–pressure space. Chymotrypsinogen, the more thermally stable of the two, is stabilized by moderate pressure at high temperatures; this stabilization is similar to the response seen in the DNA polymerases in this study. Whether this pressure stabilization is primarily due to the variation in primary structure that causes the protein to be thermostable or simply due to changes in the solvent properties of water at high temperature is not evident.

The DNA polymerases studied may have experienced any one or a combination of these stabilizing factors. Further work using well-defined biochemical systems (proteins with known structure that can be studied spectroscopically under pressure) should be able to disentangle the activation volume from the actual volume of unfolding to determine the precise interplay of inactivation mechanism, protein structure, and temperature on pressure stabilization.

In general, vegetative cells show little tolerance for temperatures above their maximum temperature for growth (Brock et al. 1994). The half-life of *P.* strain ES4 at 103°C (4°C above its optimum temperature) is 1 h (Holden and Baross 1995). At superoptimal temperatures, all organisms quickly produce heat-shock proteins which promote refolding, prevent aggregation, and degrade unrecoverable proteins (Schlesinger 1990); this response implies that protein destruction is a critical problem under heat stress. If pressure relieves this destruction, survival time at high temperatures will be increased. Holden and Baross (1995) hypothesized protein stabilization to be the cause of the instantaneous release of cultures of *P.* strain ES4 from heat stress after addition of 22 MPa pressure. The observed sta-

bilization of the DNA polymerase from this organism and potential stabilization of other enzymes could explain the thermotolerance with pressure seen in *P.* strain ES4, though the enzyme half-life within the cell may be different due to intercellular concentrations of potentially thermoprotective solutes such as salts (Hensel and Konig 1988) and low molecular weight organophosphates (e.g., Ciulla et al. 1994).

Protein stabilization as seen in the DNA polymerase could be the reason that six hyperthermophilic archaea increase their upper temperature limit for growth when under 20–40 MPa (see Introduction). Even more significant ecologically is the effect that pressure stabilization of proteins may have on a organism's enhanced ability to withstand transient temperature increases. Survival beyond the maximum temperature for growth is an important attribute in environments where temperature fluctuates over short timescales. The submarine hydrothermal flange is an example of such an environment: flanges are shelf-like metal sulfide structures that are 5–50 cm thick, with 250°–350°C vent fluid below and frigid 2°C seawater above (Delaney et al. 1992). Hyperthermophiles, including *P.* strain ES4, have been isolated from flange material where they inhabit the 80°–110°C zone in the steep thermal gradients. Because of the ambient deep-sea pressure, *P.* strain ES4 has an increased ability to survive transient variations in vent fluid temperature.

Pressure enhances the thermostability of DNA polymerases from three thermophilic organisms regardless of the native pressure of the organisms; such a pressure effect implies a positive volume of inactivation for these enzymes. This positive inactivation volume may derive from a transient volume increase during whole-protein inactivation, or perhaps from an increase in system volume during reversible protein denaturation either due to solvation of extremely hydrophobic residues or simply increased temperatures. Too little is known about the diversity and distribution of thermophiles and hyperthermophiles in the marine environment to draw any firm conclusions relating pressure stabilization to habitat or phylogeny. Pressure stabilization of proteins is a possible explanation for the enhanced thermotolerance of *P.* strain ES4 and could imply enhanced survival for thermophilic organisms at stressful temperatures under moderate pressure, increasing the fitness of these organisms in thermally variable pressurized environments such as deep-sea hydrothermal vents.

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