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ORIGINAL PAPER

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Comparison of high pressure-induced dissociation of single-stranded DNA-binding protein (SSB) from high pressure-sensitive and high pressure-adapted marine *Shewanella* species

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Abstract The effects of hydrostatic pressure on protein quaternary structure were compared for recombinant single-stranded DNA-binding protein (SSB) derived from piezosensitive, piezotolerant, and obligately piezophilic ("pressure-loving") marine *Shewanella* strains. The pressure-induced dissociation of the oligomeric SSB proteins was investigated using fluorescence anisotropy. The SSBs all exhibited striking similarity in the pressure-dependent behavior of the fluorescence intensity and emission spectrum as well as in their dissociation constants at atmospheric pressure. The free energies of subunit association into tetramers for all SSBs were between –27 and –30 kcal mol^{-1}. However, SSB from the piezosensitive *Shewanella* strain *S. hanedai* was more sensitive to pressure than that of the SSB proteins from the piezotolerant or piezophilic bacteria. The volume change of association obtained from the pressure dependence of dissociation at a fixed protein concentration (ΔV_p) for SSB from *S. hanedai* was 394–402 ml mol^{-1}. The ΔV_p values for SSB from the deeper-living *Shewanella*s were smaller and ranged from 253 to 307 ml mol^{-1}. Differences between the primary structures of the SSB proteins that could correlate with differences in sensitivity to pressure-induced dissociation were examined.

Key words Piezoadaptation · Piezophile · Pressure · Protein adaptation · *Shewanella* · Single-stranded DNA-binding protein · SSB

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Introduction

The influence of elevated pressure on the evolutionary adaptations of biological systems remains relatively poorly understood. One consequence of exposure of mesophilic organisms to elevated hydrostatic pressure may be the impairment of protein–protein and protein–nucleic acid interactions. The increase in system volumes associated with some protein–protein and protein–DNA associations is known to promote the pressure-induced dissociation of multimeric proteins from mesophiles and to inhibit protein–DNA interaction at elevated pressure (see, for example, Paladini et al. 1987; Ruan and Weber 1989; Macgregor 1990; Robinson and Sligar 1994; Royer et al. 1993; Cioni and Strambini 1997; Fujisawa et al. 1999).

Based upon comparisons of the structural and kinetic properties of homologous proteins from related organisms adapted to different pressures, it is clear that biochemical adaptation of proteins to high pressure is a hallmark feature of organisms in deep-sea environments (Swezey and Somero 1982; Hennessey and Siebenaller 1985; Gibbs and Somero 1989). To date, there has been little work addressing biochemical adaptation at the level of quaternary structure. However, hydrophobic interactions appear to be important for the pressure stabilization of many proteins from thermophiles (Hei and Clark 1994; Michels et al. 1996; Sun and Clark 2001).

In order to explore the stability of protein–protein interactions at high pressure, we chose to compare single-stranded DNA-binding protein (SSB) from three closely related marine bacteria of the genus *Shewanella* adapted to different pressure regimes. Members of the genus *Shewanella* were selected because of the wide range of pressure optima existing among strains available in culture collections (DeLong et al. 1997; Kato and Bartlett 1997; Nakasone et al. 1998; Nogi et al. 1998; Kato et al. 2000). Table 1 describes the pressure and temperature optima of the *Shewanella* strains selected for this study.

Single-stranded DNA-binding protein is an essential protein for DNA replication, recombination, and repair. *Escherichia coli* SSB is a homotetramer that binds single-

Table 1. Description of *Shewanella* strains used in this study

Strain	T_{opt}[a] (°C)	Isolation depth (m)	P_{opt}[b] (bar)	Reference
S. hanedai	20	1	1	Chilukuri and Bartlett (1997)
S. SC2A	12–18	2,000	136	Tobal (1993)
S. PT99	9	8,600	680	DeLong et al. (1997), A.A. Yayanos (unpublished)

[a]Optimum growth temperature
[b]Pressure for optimum growth at optimum temperature

stranded DNA (ssDNA) cooperatively and with high affinity, and may interact with other proteins involved in DNA metabolism (Meyer and Laine 1990). Missense mutations in the *ssb* gene are known to affect cell viability, recombination efficiency, UV-light sensitivity, and temperature sensitivity. Tetramer formation is critical to the function of SSB (Bujalowski and Lohman 1991).

We have previously described the cloning and sequencing of the *Shewanella ssb* genes (Chilukuri and Bartlett 1997). The results from this study revealed a high degree of identity in the primary structure of their gene products. Trends in the arginine/lysine ratios, in proline and glycine content, and in hydrophilicity, suggested trends in source organism temperature or pressure adaptation. In this study we document the pressure dissociation of recombinant SSB derived from three *Shewanella* strains.

Materials and methods

Buffers and chemicals

All chemicals used in protein purification were reagent grade and all solutions were prepared with distilled deionized water (MilliQ; Bedford, MA, USA). Buffers used in fluorescence studies were prepared with Ultrol grade Tris (CalBiochem, San Diego, CA, USA), EDTA puriss pa grade (Fluka Chemical, Ronkonkoma, NY, USA) and glass distilled deionized water. Guanidine HCl (ultrapure) and ssDNA cellulose were purchased from Sigma Chemical Company, St. Louis, MO, USA.

SSB proteins

SSB proteins from the three *Shewanella*s were purified from the IPTG-inducible plasmids pLC10, pLC20, and pLC40 (Chilukuri and Bartlett 1997) in *E. coli* strain BL21 (DE3) plysS (Stratagene; San Diego, CA, USA). For the overproduction of each SSB, a single Amp^r Cm^r plasmid-bearing *E. coli* colony was inoculated into L-broth containing ampicillin (100 mg l^{-1}) and grown with aeration at 37°C to an OD_{600} of 0.6. Induction of recombinant protein was then initiated by the addition of IPTG to a final concentration of 4 mM. Post-induction growth was limited to 2 h at 30°C to prevent accumulation of protein in inclusion bodies.

All purification steps were carried out at 4°C. Protein purification by polymin P precipitation and ammonium sulfate precipitation was as described by Lohman et al. (1986). The ammonium sulfate precipitate was extensively dialyzed in buffer containing 50 mM Tris, 1 mM EDTA, 25% glycerol, and 100 mM KCl (pH 7.5) and bound to ssDNA cellulose equilibrated with the same buffer. Subsequent to washing with 10 column volumes of the above solution and 5 column volumes of buffer with the KCl concentration increased to 500 mM, the protein was eluted by increasing KCl concentration to 2.5 M. Peak fractions were dialyzed in 20 mM Tris, 1 mM EDTA, 500 mM KCl, and 50% glycerol (pH 8.0) and stored at –20°C.

The purity of all three SSB proteins was greater than 95%, as determined by polyacrylamide gel electrophoresis and staining with Coomassie Blue. Comparison of arginine ratios calculated from the deduced amino acid sequences with the ratios obtained from amino acid analysis gave purity estimates of more than 97% for all three proteins. Amino acid analysis of the purified proteins was conducted by the Protein and Nucleic Acid Facility at Stanford University Medical Center, Stanford, CA, USA. The level of contamination of the *Shewanella* SSB proteins by wild type *E. coli* SSB was determined to be less than 1% by polyacrylamide gel electrophoresis and Western blot using antibody raised against *E. coli* SSB (generously provided by C. Urbanke). The extinction coefficients at 280 nm were calculated by the method of Pace et al. (1995) from the tryptophan, phenylalanine, and tyrosine content of the proteins. The extinction coefficients for the monomeric SSB proteins were: *S. hanedai* SSB $\varepsilon = 3.85 \times 10^4\ M^{-1}\ cm^{-1}$, *S.* SC2A SSB $\varepsilon = 3.7 \times 10^4\ M^{-1}\ cm^{-1}$, *S.* PT99 SSB $\varepsilon = 3.4 \times 10^4\ M^{-1}\ cm^{-1}$, and *E. coli* SSB $\varepsilon = 2.8 \times 10^4\ M^{-1}\ cm^{-1}$. Protein concentration was determined from the absorbance at 280 nm corrected for light scattering measured by the absorbance at 330 nm (Leach and Scheraga 1960).

Fluorescence measurements

Fluorescence measurements were made by photon counting with an ISS K2 multifrequency phase fluorometer using a 300 W xenon light source. High-pressure anisotropies were measured with the L-format, using a custom-built pressure bomb similar to that described by Paladini and Weber (1981). Pressure units used here are given in bar to be consistent with other related high-pressure biophysical studies

(1 atmosphere = 1.01325×10^5 Pa = 1.01325 bar). The anisotropy data were corrected for instrumental polarization and for pressure-induced window birefringence using the equations of Paladini and Weber (1981) and polarization measurements as a function of pressure of *N*-acetyl-L-tryptophanamide in glycerol at 4°C. Excitation wavelength was 295 nm with a 4-nm bandpass. A Hoya U340 bandpass filter was placed in the excitation path to eliminate stray light. Single-point emission intensities were measured through a UV34 filter. Emission spectra were determined with an 8-nm bandpass. All spectra are technical and are not corrected for the wavelength-specific response of the photomultiplier or the monochromator. The temperature of the samples was maintained at 25.0 ± 0.1°C.

In general, the acquisition time was set so that a minimum of 100,000 counts were acquired. The fluorescence intensity of the buffer in the absence of protein was less than 4% of the protein emission intensities at all protein concentrations and was ignored in anisotropy calculations. The samples were allowed to equilibrate for 10 min after each increase or decrease in pressure before fluorescence measurements were taken. Anisotropy values did not change with longer periods, indicating that 10 min was sufficient to establish equilibrium. The anisotropies measured at 1 bar and high protein concentration were taken to correspond to the oligomer. The relative quantum yields of fluorescence for the oligomeric and dissociated proteins were measured from the ratio of the fluorescence intensities at 1 bar and 2 kbar.

The SSB proteins were dialyzed in a 4,000-fold excess volume of buffer over a period of 3 h prior to making fluorescence measurements. The data for each protein concentration represent two or more separate experiments made with different dialysis preparations for each SSB protein.

Theoretical considerations

Elevated pressure promotes the dissociation of oligomeric proteins. The degree of dissociation can be determined from the anisotropy measured at different pressures by the equation

$$\alpha_p = \left(1 + Q\left(r_p - r_M\right)/\left(r_O - r_p\right)\right)^{-1} \quad (1)$$

where α_p is the degree of dissociation at pressure p, r_p is the anisotropy at pressure p, r_M and r_O are the anisotropy of the monomer and the oligomer, respectively, and Q is the ratio of the quantum yields of fluorescence of the monomer and the oligomer (Paladini and Weber 1981). The dissociation constant for the oligomer as a function of the concentration and degree of dissociation of the protein is defined by

$$K_d^{(P)} = N^N C^{N-1} \alpha^N / (1 - \alpha) \quad (2)$$

where N is the number of subunits and C is the concentration of protein as the oligomer (Erijman and Weber 1991). For a tetramer in equilibrium with monomers, in the absence of intermediates such as dimers,

$$K_d^{(P)} = 256 T_0^3 \alpha^4 / (1 - \alpha) \quad (3)$$

where T_0 is the molar concentration of the total protein as tetramer. The pressure dependence of the dissociation constant K_d is given by

$$K_d^{(P)} = K_d^{(1bar)} \exp\left(P\Delta V / RT\right) \quad (4)$$

where $K_d^{(P)}$ and $K_d^{(1\ bar)}$ are the dissociation constants at pressure P and atmospheric pressure, respectively, ΔV is the volume change upon oligomer association, R is the gas constant, and T is the absolute temperature. Combining Eqs. 3 and 4, it is possible to derive

$$\ln\left(\alpha_p^4 / \left(1 - \alpha_p\right)\right) = \ln K_d^{(1bar)} / 256 T_0^3 + P\Delta V / RT \quad (5)$$

Thus, the standard volume change (ΔV) of tetramer association (in ml mol^{-1}) and the dissociation constant ($K_d^{(1\ bar)}$) at atmospheric pressure can be calculated from measurements of α versus pressure. The value of ΔV obtained by this method is denoted ΔV_p, obtained from the change in the degree of dissociation, as a function of pressure at a fixed protein concentration.

Two additional parameters can be calculated from $K_d^{(1\ bar)}$, the concentration ($C_{1/2}$) at which $\alpha = 0.5$ at atmospheric pressure, and ΔG, the change in Gibbs free energy upon monomer association. For a tetramer–monomer equilibrium:

$$C_{1/2} = \left(K_d^{(1bar)} / 32\right)^{1/3} \quad (6)$$

$$\Delta G = RT \ln K_d^{(1bar)} \quad (7)$$

Results

Pressure affects the intrinsic fluorescence intensities, anisotropies, and emission spectra of *Shewanella* SSB proteins

The effect of pressure on the fluorescence emission spectrum and intensity, and fluorescence anisotropy for SSB from *S. hanedai* are shown as a prototype for the behavior of all three *Shewanella* SSBs. SSB proteins from the other *Shewanella*s behaved similarly, albeit with quantitative differences. Figure 1A shows normalized spectra of the intrinsic fluorescence emission at 1 bar and 2 kbar for SSB from *S. hanedai*. Maximal emission at 1 bar was close to 356 nm, indicating a polar environment for the tryptophan residues in the native protein. A red shift of about 2 nm at 2 kbar was observed consistently for all the *Shewanella* SSB proteins. The small spectral displacement suggests that the environment of the tryptophan residues changed little with an increase in pressure.

The effects of pressure on the intrinsic fluorescence intensity and on the decrease in fluorescence anisotropy of *S. hanedai* SSB are shown in Fig. 1B. The fluorescence anisotropy decreased with pressure in the range of 1 bar to 1 kbar and reached a plateau at higher pressures. The fluo-

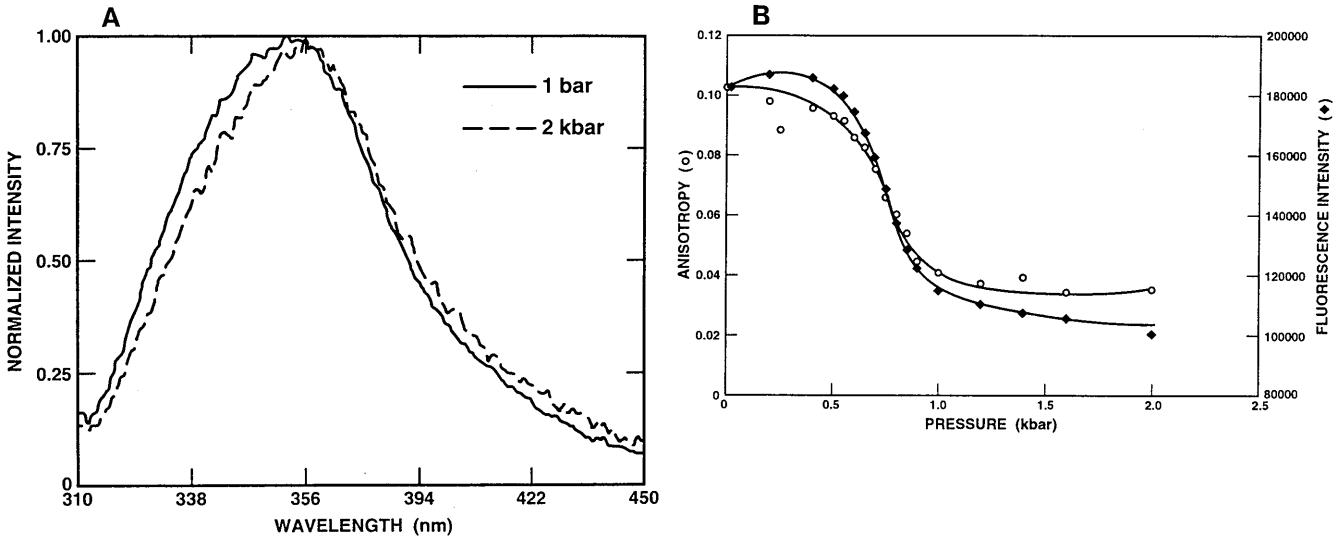

Fig. 1. **A** Effect of pressure on the intrinsic fluorescence emission spectrum of *S. hanedai* SSB. Fluorescence was measured for 7 μM (monomer) SSB in 10 mM Tris, 0.1 mM EDTA, and 200 mM NaCl, pH adjusted to 8.1 at 25°C. The *solid line* shows the emission at 1 bar and the *dashed line* shows the spectrum at 2 kbar hydrostatic pressure. The excitation wavelength was 295 nm. **B** Effect of pressure on the fluorescence intensity and anisotropy of *S. hanedai* SSB. Protein concentration and buffer conditions as shown for Fig. 1A. *Filled squares* indicate intrinsic fluorescence intensity and *open circles* indicate anisotropy calculated from polarization values corrected for window birefringence

Table 2. Oligomer and monomer anisotropies of the *Shewanella* single-stranded DNA-binding proteins (SSBs)

SSB source	Oligomer anisotropy[a]	Monomer anisotropy[b]	Ratio of intensity (Q)[c]
S. hanedai	0.101 ± 0.002	0.033 ± 0.004	0.71
S. SC2A	0.102 ± 0.003	0.035 ± 0.006	0.69
S. PT99	0.097 ± 0.005	0.031 ± 0.006	0.79

[a]Determined from the anisotropy values at 1 bar, 25°C at the high protein concentration
[b]Determined from the anisotropy values at high pressure and 1 μM monomer protein concentration
[c]Measured from the ratio of fluorescence intensities of the SSBs at 1 bar and 2 kbar

rescence intensity also decreased with increasing pressures and corresponded with the decrease in anisotropy over the same pressure range. A small increase in fluorescence intensity was observed between 1 and 400 bar. Decreases in anisotropy and intensity and red shift of the emission with pressure have been reported for several oligomeric proteins, including the *lac* repressor, yeast hexokinase, and β_2-tryptophan synthase, and indicate subunit dissociation (King and Weber 1986; Royer et al. 1986; Silva et al. 1986; Ruan and Weber 1988).

Pressure-induced dissociation of *Shewanella* SSB proteins

The pressure dependence of the oligomer-monomer equilibrium was compared for the three *Shewanella* SSB proteins.

Fluorescence anisotropy measurements were made for each SSB protein at pressures ranging from 1 bar to 2 kbar. The anisotropy values measured at 1 bar at the higher protein concentration for each SSB were taken to be the anisotropy of the native oligomer. The minimum anisotropy values measured at high pressure were taken to represent the anisotropy of the monomer. Table 2 shows the oligomer and monomer anisotropy values measured at 25°C for the *Shewanella* SSB proteins. The degree of dissociation as a function of pressure for each *Shewanella* SSB was calculated using Eq. 1 from the observed anisotropies at different pressures and the anisotropy values for the oligomer and the monomer shown in Table 2.

Volume changes of association and dissociation constants for oligomeric proteins were obtained through analysis of the pressure-dependent dissociation curves. The calculated values of the degree of dissociation for each *Shewanella* SSB were analyzed based on tetramer, trimer, and dimer models for an oligomer–monomer equilibrium. Several features of the *Shewanella* SSB proteins were consistent with the premise that the proteins are tetrameric in their native states. Based on the size of the *Shewanella* SSB monomers (24.7–26.3 kDa) (Chilukuri and Bartlett 1997), the molecular weight would be predicted to be 99–105 kDa for tetramers and 74–79 kDa for trimers. All three *Shewanella* SSB proteins were retained on microconcentrator filters with a molecular weight cutoff of 100 kDa. The threefold decrease in anisotropy resulting from an increase in pressure or upon dilution suggests an oligomer of three or more subunits. At the level of primary sequence, all three *Shewanella* SSB proteins exhibit greater than 50% identity with the SSB from *E. coli* and other bacteria whose tetrameric nature has been established by the crystal structure (Raghunathan et al. 1997; Yang et al. 1997), by ultracentrif-

ugation (Genschel et al. 1996), or by their ability to form cross-species heterotetramers (DeVries et al. 1994). A tetramer–monomer equilibrium was assumed for the calculation of volume changes of association (ΔV) and the dissociation constants ($K_d^{(1\ bar)}$) of the *Shewanella* SSB proteins. The values for the degree of dissociation as a function of pressure were analyzed using Eq. 5.

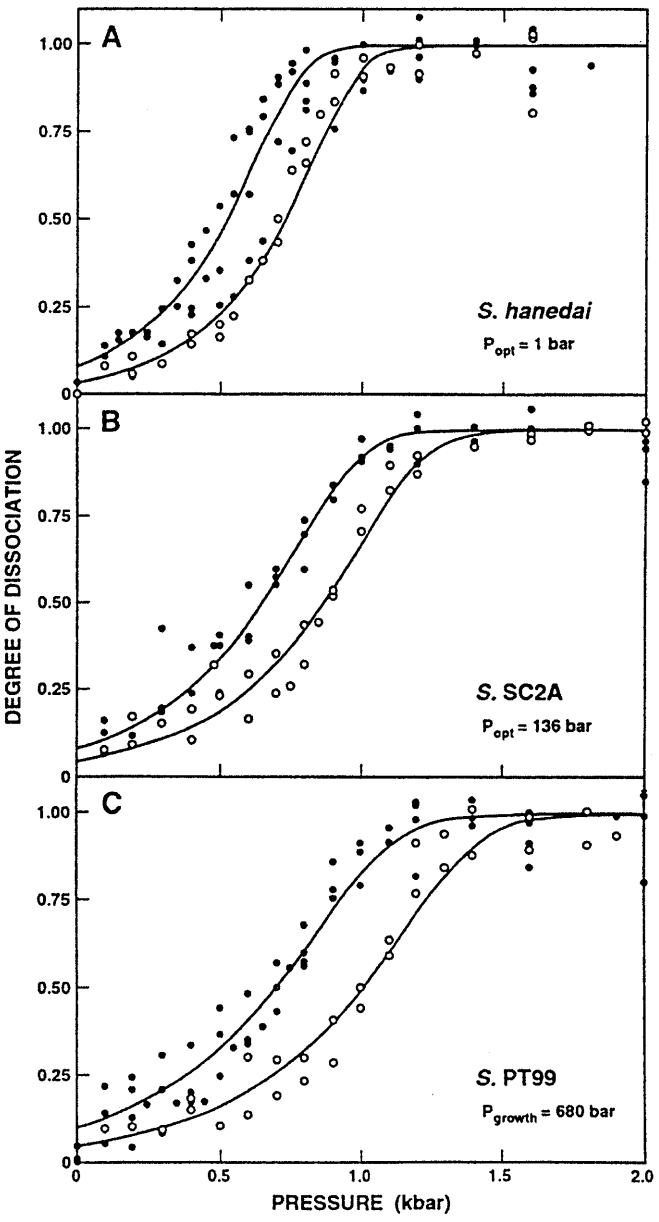

Fig. 2A–C. Dissociation of *Shewanella* SSB by pressure. SSB from *S. hanedai* (**A**), *S.* SC2A (**B**), and *S.* PT99 (**C**) at two concentrations were exposed to increased pressure at 25°C and the degree of dissociation was calculated from the fluorescence anisotropies using Eq. 3. In each panel, the *filled circles* indicate data for the lower concentration (1 μM monomer) and the *open circles* indicate data for the higher concentration (7 μM monomer; 4 μM for *S.* SC2A SSB). The *solid lines* are theoretical dissociation curves for the lower and higher protein concentrations derived from the $K_d^{(1\ bar)}$ and ΔV_p values shown in Table 3

The pressure-dependence of α, the degree of dissociation, for two concentrations of *Shewanella* SSB oligomers are shown in Fig. 2A–C. The lower protein concentrations employed in this study (about 1 μM monomer) are within the range (0.5–1 μM monomer) calculated for *E. coli* SSB in vivo (Williams et al. 1984). Pressure-dependent dissociation was observed for all the *Shewanella* SSB proteins. The shift in the curves to higher pressures with an increase in protein concentration observed for the *Shewanella* SSB proteins is consistent with the dissociation of the oligomer by pressure. The measured values of SSB dissociation at either concentration were in excellent agreement with the dissociation curve predicted by the tetramer model.

The volume change of association calculated from the pressure dependence of dissociation at a fixed concentration is denoted ΔV_p and ranged from 253 ml mol^{-1} for SSB from the piezophilic strain (*S.* PT99) to 402 ml mol^{-1} for SSB from the piezosensitive *S. hanedai*.

The pressure at which an oligomeric protein is 50% dissociated, $P_{1/2}$, is a means of comparing the pressure stabilities of SSB proteins from the different species and varied from 520 bar for SSB from the piezosensitive strain *S. hanedai* to 750 bar for SSB from the extremely piezophilic strain *S.* PT99 at concentrations close to 1 μM monomer, shifting to higher pressures at the higher concentrations.

The dissociation constant $K_d^{(1\ bar)}$ is conveniently expressed as $C_{1/2}$, the protein concentration at which $\alpha = 0.5$ at 1 bar (Eq. 6). The calculated $C_{1/2}$ for the experiments in Fig. 2 are shown in Table 3. The $K_d^{(1\ bar)}$ or $C_{1/2}$ values reflect the subunit affinity at 1 bar and were similar for all SSB proteins. Values for the free energy of association at 1 bar (ΔG), ranged from –27 to –30 kcal mol^{-1} for all of the *Shewanella* SSB proteins.

Discussion

The main objective of this study was to determine the relationship, if any, between bacterial pressure adaptation and the stability of an oligomeric protein as a function of pressure. The pressure dissociation of SSB proteins derived from three *Shewanella* strains differing in pressure adaptation was analyzed.

SSB from the piezosensitive *Shewanella* strain was the most sensitive to dissociation by pressure of the three SSB proteins examined and this was reflected in the measured volume changes of association. The ΔV of *S. hanedai* SSB was consistently the largest value observed for the *Shewanella* SSB proteins with ΔV_p = 394–402 ml mol^{-1} (Table 3). SSB proteins from the deeper-living *Shewanella* strains exhibited lower volume changes ranging from ΔV_p = 253 to 307 ml mol^{-1}. The $P_{1/2}$ of SSB from *S.* PT99 was the highest among the *Shewanella* SSB proteins for any comparison at similar protein concentrations (Table 3). Although the *Shewanella* strains are differently adapted to temperature, the differences in pressure sensitivities and in volume changes of association cannot be attributed to the temperature at which the measurements were made, since

Table 3. Estimated volume changes of association, dissociation constants at atmospheric pressure, and standard free energies of association for the *Shewanella* SSB proteins at 25°C calculated using the model for a tetramer–monomer equilibrium

SSB source	Concentration (μM)[a]	ΔV_p^b (ml mol^{-1})	$P_{1/2}$ (kbar)	$K_d^{(1\ bar)}$ (M)	$C_{1/2}$ (nM)	ΔG (kcal mol^{-1})
S. hanedai	1	394 ± 23	0.52	1.26×10^{-22}	16 ± 2	–29.9 ± 0.3
	6.9	402 ± 18	0.73	1.29×10^{-21}	34 ± 5	–28.5 ± 0.3
S. SC2A	1	307 ± 17	0.65	1.64×10^{-22}	17 ± 2	–29.7 ± 0.3
	3.9	296 ± 21	0.87	9.6×10^{-22}	31 ± 6	–28.7 ± 0.4
S. PT99	1	253 ± 18	0.75	3.4×10^{-22}	22 ± 4	–29.3 ± 0.3
	7.1	254 ± 22	1.005	6.1×10^{-21}	58 ± 13	–27.6 ± 0.4

[a]Expressed as monomer
[b]Calculated from the pressure dependence of the dissociation at a fixed protein concentration

similar patterns were observed at 5° and 25°C (data not shown). Previous work with animals has suggested that the deep-sea environment selects for improved enzyme function and protein stability at high pressure (Siebenaller and Somero 1978; Somero and Siebenaller 1979; Swezey and Somero 1982).

There is great similarity among the *Shewanella* SSB proteins with regard to their emission spectra, and the pressure dependence of the decrease in fluorescence intensity and anisotropy. The $K_d^{(1\ bar)}$ values, and consequently the ΔG values, are not significantly different among the *Shewanella* SSB proteins (Table 3). The free energies of association range from –27 to –30 kcal mol^{-1} and are comparable to ΔG values calculated for other tetrameric proteins such as lactate dehydrogenase and glyceraldehyde phosphate dehydrogenase (King and Weber 1986; Ruan and Weber 1988).

What are the factors that determine the extent of the pressure resistance of each of the SSB proteins? Relatively small changes, of the order of one hydrophobic interaction, may have a large impact on protein stability (Matthews 1993). The *E. coli* SSB tetramer has previously been shown to be resistant to dissociation by dilution and thermally stable. However, a single amino acid substitution (H55Y) results in dramatic differences in the subunit affinity and temperature stability (Bujalowski and Lohman 1991).

The crystal structure of the human mitochondrial SSB (*Hs*mt SSB) and the DNA-binding portion of the *E. coli* SSB (Raghunathan et al. 1997; Yang et al. 1997) indicate that the tetramers are dimers of dimers. Both in the case of SSB monomer–monomer interactions in the dimer, and dimer–dimer contacts in the tetramer, six-stranded β-sheet interactions form the subunit interfaces. Association primarily results from hydrogen-bonding; however, a salt bridge also exists between the dimers. Genetic experiments have confirmed that the six-stranded β-sheet is an important interface for SSB tetramerization. His-55, which is important for monomer–monomer interaction, is present in all the *Shewanella* SSBs as well as the Lys-7 and Glu-80 residues needed for the dimer–dimer salt bridge.

Trends in the amino acid composition of the *Shewanella* SSB proteins that are suggestive of pressure and temperature adaptation of the *Shewanella* strains were previously noted (Chilukuri and Bartlett 1997). Within the conserved amino- and carboxy-terminal regions of the protein, the *Shewanella* SSB proteins display a remarkable similarity in amino acid composition, with only three unconserved amino acid substitutions. The most striking differences between the primary structure of the *Shewanella* SSB proteins are related to the size and composition of the central variable region. There is a reduction in the glycine and proline composition which accompanies increasing pressure optimum of the source strain (Chilukuri and Bartlett 1997). The *S. hanedai* protein has a nearly twofold greater proline and glycine content overall than the SSB from *S*. PT99. In addition to P48 in the conserved portion of the *S. hanedai* SSB protein, the majority of the additional proline residues are located in the central variable region of the protein. A reduction in helix-breaking (proline) and helix-destabilizing (glycine) residues may reduce the flexibility of SSB from *S*. PT99. A correlation between low flexibility and low compressibility has been documented for globular proteins (Gross and Jaenicke 1994). In addition, a proline to glycine substitution in staphylococcal nuclease has been reported which both increased the stability of the protein to elevated pressure as well as decreased chain mobility, perhaps reflecting greater compression in the folded state (Royer et al. 1993).

The *E. coli* SSB tetramer has been shown to bind ssDNA with far greater affinity than monomers. The relative pressure sensitivities of DNA binding have yet to be established for any SSB proteins. Such binding activity is likely to result in the exclusion of previously electrostricted water molecules into the bulk solution and may be associated with a large system volume change for proteins which do not have to function at high pressure. The DNA-binding activity of the restriction endonuclease *Eco*RI is associated with a volume change of +82 ml mol^{-1} (Macgregor 1990). Analyses of *E. coli ssb* mutants bearing deletions in the variable region suggest that the length of this region affects modulation of DNA-binding activity. Thus, the examination of *Shewanella* SSB DNA binding activities could reveal an additional characteristic of their biochemical adaptation to high pressure.

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