Salt Bridges in the Hyperthermophilic Protein Ssh10b Are Resilient to Temperature Increases*

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Hyperthermophiles are a group of microorganisms with an optimum growth temperature between 80 and 100 °C. Remarkably, hyperthermophilic proteins and their mesophilic homologues typically show 40–85% sequence similarity, and their three-dimensional structures are highly superimposable (1–4). These facts indicate that the molecular basis of their extreme thermal tolerance is associated with the delicate balance of non-covalent interactions.

Considerable efforts have been invested in recent years to understand how proteins from hyperthermophiles can maintain stability at high temperatures. Several factors, such as improvement in packing density, strengthening of hydrophobic cores, and decreased length of surface loops, are thought to correlate with the increased thermostability of hyperthermophilic proteins (5–9). The most commonly cited feature, however, is an increased number of conserved salt bridges in a temperature-dependent fashion (29). The crystal structure of Ssh10b reveals that the monomer contains four isolated salt bridges: Glu-36/Lys-68, Asp-63/Lys-97, Glu-66/Arg-95, and an ion-pair network, Lys-40/Glu-91/Arg-71/Glu-69 (30), evidently more than the average of about 5 salt bridges per 150 amino acid residues (31). However, experimental estimates of the free energy contribution of salt bridges to protein stability have led to conflicting conclusions, ranging from them having a stabilizing, insignificant, or even a destabilizing effect (17–23). The association of two charged residues to form a salt bridge is thought to incur a substantial desolvation penalty that is seldom completely compensated for by favorable interactions within the salt bridge and the rest of the protein (24–26). A continuum solvation model was used to investigate how this same argument applies at the high temperatures hyperthermophiles experience and predicted that at such high temperatures the desolvation penalty for the formation of a salt bridge is markedly reduced (25). Furthermore, molecular simulations suggest salt bridges are extremely resilient to temperature increases and thus are specially suited to promoting protein stability at high temperatures (26). However, attempts to obtain thermodynamic information at high temperature have been plagued by irreversibility of unfolding and/or inaccessibility of the unfolding transition to physical measurements. So far there are no reports of experiments to measure the stability contribution of salt bridges at high temperatures and to provide evidence for these theoretical predictions.

The DNA-binding protein Ssh10b from the archaeon Sulfolobus shibatae is a member of the Sac10b family that is thought to be involved in chromosomal organization or DNA repair/recombination. Ssh10b is a highly thermostable dimeric protein composed of two identical subunits, each monomer consisting of 97 amino acid residues with no disulfide bonds (27, 28). Ssh10b constitutes about 4–5% of total cellular protein, and binds dsDNA without apparent sequence specificity. Ssh10b is also capable of constraining negative DNA supercoils in a temperature-dependent fashion (29). The crystal structure of Ssh10b reveals that the monomer is a mixed α/β structure comprised of four β-strands and two α-helices (Fig. 1), each monomer containing four isolated salt bridges: Glu-36/Lys-68, Glu-54/Arg-57, Asp-63/Lys-97, Glu-66/Arg-95, and an ion-pair network, Lys-40/Glu-91/Arg-71/Glu-69 (30), evidently more than the average of about 5 salt bridges per 150 amino acid residues (31). However, only the Glu-36/Lys-68 and Glu-54/Arg-57 salt bridges are highly conserved across the Sac10b family (Fig. 2). All of these salt bridges and ion-pair networks are surface-exposed, located on the back of the Ssh10b dimer.
Expression plasmids of Ssh10b and its variants were transformed into the *Escherichia coli* BL21 (DE3) host strain. For protein expression, a single colony was grown in 100 ml of LB media containing 100 μg/ml ampicillin by shaking (~220 rpm) at 37 °C overnight. Cultures were diluted 1:50 in fresh LB antibiotic-containing media and shaken for about 3 h at 37 °C. Target protein expression was then induced at $A_{600} = 0.8–1.0$ by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.3 mM, and growth was allowed to continue for about 5 h at 37 °C with constant shaking.

After harvesting by centrifugation (4,000 rpm for 30 min), cell pellets from 1-liter cultures were re-suspended in 25 ml of buffer A (20 mm Tris–HCl, pH 7.5) and then disrupted by ultrasonication on ice. The lysate was maintained at 60 °C for 20 min to precipitate the *E. coli* proteins, and then centrifuged at 16,000 rpm for 30 min at 4 °C. The supernatant was applied to a 20-ml Source 30S column, which had been equilibrated with buffer A, and proteins were eluted with a 0–50% gradient (120 ml) of buffer B (1.5 M NaCl, 20 mm Tris-HCl, pH 7.5). Fractions containing the target protein were identified by 15% SDS-PAGE, dialyzed overnight against deionized water, and then lyophilized.

**Unfolding Studies**—Unfolding of Ssh10b and its variants was studied by taking circular dichroism (CD) measurements with a $\pi^*$-pistar 180 spectrometer (Applied Photophysics Ltd, UK), performed in buffer H (10 mm HEPES, pH 7.0), at a protein monomer concentration of about 25 μM. The CD signal was monitored using a rectangular quartz cuvette with a path length of 1 mm. For urea-induced unfolding, the urea solution was freshly prepared on the day of use. The samples containing various concentrations of urea were equilibrated at 25 °C overnight and then measured by far-UV CD at 222 nm. For heat-induced unfolding, each sample was heated from 40 to 98 °C and then cooled from 98 to 40 °C using stepwise changes of 2 °C, and the CD signal was recorded after equilibration for 2 min at each temperature point. All unfolding experiments were repeated 3–4 times.

**Analysis of the Denaturation Data**—Previous studies in our laboratory have shown that both denaturant- and heat-induced unfolding of Ssh10b are fully reversible and follow a two-state mechanism involving a native dimer and two denatured monomers (28). Therefore, in this work the thermodynamic properties of Ssh10b and its variants were calculated assuming a two-state denaturation process in Reaction 1.

$$K_{\text{eq}} = \frac{[\text{N}]^2}{[\text{U}]^2}$$

**REACTION 1**

The observed equilibrium constant ($K_{\text{obs}}$) and the corresponding free energy change ($\Delta G$) at temperature $T$ or denaturant concentration $[D]$ were calculated according to Equations 1 and 2 (28),

2 The abbreviation used is: DMC, double mutant cycle.
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**FIGURE 2. Sequence alignment of archeal Ssh10b homologues.** Ssh (S. shibatae), Sso (S. solfataricus), Sac (S. acidocaldarius), Afu (Archeoglobus fulgidus), Hbu (Hyperthermus butylicus), Mja (Methanocaldococcus jannaschii), Mka (Methanopyrus kandleri), Mst (Methanosphaera stadtmanae), Mth (Methanoseta thermophila), Pab (Pyrococcus abyssi), Pfu (Pyrococcus furiosus), Pho (Pyrococcus horikoshii), Pko (Pyrococcus kodakaraensis), Uncm (Uncultured methanogenic archaeon RC-1), Tac (Thermoplasma acidophilum), Pae (Pyrococcus aerophilum). The four residues involved in the two conserved salt bridges are marked with plus signs. The figure was rendered using the program DNAMAN.

\[
K_{obs} = \frac{2 \times P_i}{y_0 + m_i[D] - y_0 + m_i[D]} \times \frac{[y_0 + m_i[D] - y]^2}{-y - y_0 + m_i[D]} \quad (\text{Eq. 1})
\]

\[
\Delta G = -RT \ln(K_{obs}) = \Delta G(H_2O) - m_o[D] \quad (\text{Eq. 2})
\]

where \(P_i\) is the total protein concentration in monomer units; \(R\) is the gas constant; \(T\) is the absolute temperature; \(y\) is the experimentally measured signal value at a given temperature (\(T\)) or given denaturant concentration ([\(D\)]); \(y_0\) and \(y_1\) are the intercepts; and \(m_o\) and \(m_i\) are the slopes of the native and unfolded baselines, respectively.

According to the linear free energy model (37–40), changes in free energy (\(\Delta G\)) that occur on unfolding are expected to vary linearly with denaturant concentration in Equation 3 ([\(D\)]),

\[
\Delta G = \Delta G(H_2O) - m_o[D] \quad (\text{Eq. 3})
\]

where \(\Delta G(H_2O)\) represents the free energy change of unfolding in the absence of denaturant and \(m_o\) is the slope of the transition for the free energy.

For thermal unfolding, assuming that the heat capacity change (\(\Delta C_p\)) between the native and unfolded states of the system is relatively independent of temperature, gives us Equation 4 (41, 42),

\[
\Delta G(T) = -RT \ln(K_{obs}) = \Delta H_m - T \Delta S_m + \Delta C_p \left( T - T_m \right) + T \ln \left( \frac{1 - (T - T_m)}{T} \right) \quad (\text{Eq. 4})
\]

where \(\Delta H_m\) and \(\Delta S_m\) are the enthalpy and entropy changes, respectively, of the protein at the transition midpoint, where \(T = T_m\). Within the transition range, where \(T \times \ln(1 - (T - T_m)/T) \sim T_m - T\), Equation 4 can be simplified to the van’t Hoff plot in Equation 5.

\[
\Delta G(T) = -RT \ln(K_{obs}) = \Delta H_m - T \Delta S_m \quad (\text{Eq. 5})
\]

The temperature of the transition midpoint (\(T_m\)) can be calculated according to Equation 6.

\[
T_m = \frac{\Delta H_m}{\Delta S_m - R \times \ln(P_i)} \quad (\text{Eq. 6})
\]

The uncertainties of the data were represented as two-sided 95% confidence intervals, which are given by Equation 7,

\[
b \pm t_{0.975} \times se(b) \quad (\text{Eq. 7})
\]

where \(b\) is the data (\(\Delta G, \Delta H_m\), and \(T_m\)), etc., \(se(b)\) is the standard error, \(t_{0.975}\) is the value of the \(t\) distribution with the number of degrees of freedom \(v\) for the two-sided 95% confidence interval.

The direct stability contribution of each salt bridge was represented by using the average values of the coupling free energy of the four different DMCs and uncertainties estimated by Equation 7.

**RESULTS**

**Mutagenesis, Expression, and Purification of Ssh10b and Its Variants**—Eight mutants for each salt bridge (Ala-36/Lys-68, Gln-36/Lys-68, Glu-36/Ala-68, Ala-36/Leu-68, Ala-36/Leu-68, Gln-36/Ala-68, and Gln-36/Leu-68) were constructed by site-directed mutagenesis. Wild-type Ssh10b and its variants were overexpressed in *E. coli* BL21 (DE3) and purified to homogeneity. Protein purity was higher than 95% as confirmed by 15% SDS-PAGE.

**Unfolding Studies**—Ssh10b is resistant to urea-induced denaturation in phosphate buffer (28), but is more susceptible to urea-induced denaturation in monovalent ion buffers (43). And for any one of above proteins, when the urea-denatured protein solution was diluted to a lower concentration of denaturant, the CD spectrum of the renatured protein was identical to that of the native protein, indicating that the urea-induced unfolding processes of Ssh10b and its variants are fully reversible (data not shown). Fig. 3 shows representative urea-induced unfolding profiles for Ssh10b and its partial variants in buffer H. The linear free energy model was used to analyze the urea-induced unfolding profiles. Results are presented in Table 1 and reveal that the difference in stability between different variants is marked: relative to the Ssh10b wild-type, some variants show a decrease in stability free energy at 298 K (\(\Delta G(298)\)) of up to 7 kJ/mol, while others show a decrease in \(\Delta G(298)\) of more than 10 kJ/mol.
far-UV CD at 222 nm are nearly superimposable, and for each one the CD spectrum of the refolded protein is nearly identical to that of the native protein (data not shown), indicating that the processes of heat-induced unfolding of Ssh10b and its variants are fully reversible. Fig. 4 shows representative heat-induced unfolding profiles for Ssh10b and its partial variants in buffer H. The heat-induced unfolding profiles of Ssh10b and its variants were analyzed by using the two-state denaturation model described under “Experimental Procedures”; the parameters $\Delta H_m$ and $\Delta S_m$ were obtained by fitting $K_{obs}$ to the van’t Hoff plot, the parameter $T_m$ was then obtained by substituting $\Delta H_m$ and $\Delta S_m$ into Equation 6. The $T_m$ values of these variants were all around 353 K, and so the stability free energy values of these proteins at 353 K ($G_{(353)}$) were calculated using the van’t Hoff plot (Table 1). As with the results for urea-induced unfolding, the difference between different variants in stability free energy at 353 K was also marked: the difference in $G_{(353)}$ between mutants and the wild-type ranged from −8.3 to 7.5 kJ/mol.

**Estimating the Contribution of Salt Bridges to Stability by Using DMC Analysis** — DMC analysis was used to estimate the strength of the two conserved salt bridges (Glu-36/Lys-68 and Glu-54/Arg-57) in protein Ssh10b. Fig. 5 shows a general scheme for a DMC consisting of two single and one double mutant. A thermodynamic cycle is set up between the wild type, each of the single mutants, and the double mutant. The effects of single amino acid substitutions of each residue involved in the salt bridge and the effect of simultaneous replacement at both positions on the stability of the protein were measured. If the two charged residues do not interact with each other, the effect of substitution of either of the two residues will be independent of the replacement of the other, in other words, the effect on protein stability resulting from their simultaneous replacement will be equal to the sum of the effects of the two single mutations. By contrast, if the two residues do interact with each other, the effect of substituting either of the two residues will depend on the substitution of the other. By using the DMC method, the so-called coupling free energy of a salt bridge can be obtained. As shown in Fig. 5, the coupling free energy ($\Delta G_{coup}$) is defined in Equations 8–11 (44).

$$\Delta G_{coup} = \Delta G_{wt \to dm} - \Delta G_{wt \to m1} - \Delta G_{wt \to m2}$$  
(Eq. 8)

where,

$$\Delta G_{wt \to dm} = \Delta G_{dm} - \Delta G_{wt}$$  
(Eq. 9)

$$\Delta G_{wt \to m1} = \Delta G_{m1} - \Delta G_{wt}$$  
(Eq. 10)

$$\Delta G_{wt \to m2} = \Delta G_{m2} - \Delta G_{wt}$$  
(Eq. 11)

So Equation 8 can be simplified to Equation 12.

$$\Delta G_{coup} = \Delta G_{dm} - \Delta G_{m1} - \Delta G_{m2} + \Delta G_{wt}$$  
(Eq. 12)

Here, because Ssh10b is a homodimer, the coupling free energy for each salt bridge in the molecule can be represented as Equation 13.

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**TABLE 1**

Parameters obtained from the analysis of unfolding experiments

$G_{(353)}$ was calculated from the results of urea-induced unfolding, and other parameters were calculated from the results of heat-induced unfolding. The uncertainties of the data were estimated according to Eq. 7.

| Proteins          | $\Delta G_{(353)}$ | $\Delta H_m$ | $\Delta S_m$ | $T_m$ | $G_{(353)}$ |
|-------------------|-------------------|--------------|--------------|-------|-------------|
| Wild type         | 51.1 ± 0.7        | 379.8 ± 15.9 | 985.1 ± 45.1 | 353.9 ± 0.2 | 32.1 ± 0.3 |
| A36/K68           | 36.0 ± 0.4        | 304.5 ± 10.2 | 795.1 ± 29.5 | 344.8 ± 0.2 | 22.8 ± 0.2 |
| Q36/K68           | 39.7 ± 0.5        | 348.1 ± 12.8 | 1021.0 ± 36.7 | 348.7 ± 0.2 | 26.8 ± 0.3 |
| E36/A68           | 46.8 ± 0.6        | 384.0 ± 13.9 | 996.4 ± 39.3 | 354.1 ± 0.2 | 32.2 ± 0.3 |
| E36/L68           | 53.1 ± 0.7        | 332.0 ± 14.2 | 848.1 ± 40.0 | 354.6 ± 0.2 | 32.6 ± 0.3 |
| A36/L68           | 43.6 ± 0.6        | 318.3 ± 14.4 | 806.5 ± 40.8 | 355.8 ± 0.3 | 33.6 ± 0.3 |
| A36/L68           | 54.9 ± 0.7        | 402.8 ± 17.8 | 1042.1 ± 50.0 | 356.4 ± 0.2 | 35.0 ± 0.4 |
| Q36/L68           | 47.7 ± 0.5        | 486.0 ± 22.0 | 1264.5 ± 64.0 | 359.3 ± 0.2 | 39.6 ± 0.4 |
| Q36/L68           | 58.4 ± 0.8        | 458.1 ± 17.9 | 1186.9 ± 50.0 | 359.3 ± 0.2 | 39.1 ± 0.4 |
| Q54/R57           | 42.0 ± 0.7        | 331.5 ± 11.5 | 856.3 ± 32.9 | 351.0 ± 0.2 | 29.2 ± 0.3 |
| Q54/R57           | 42.1 ± 0.6        | 297.1 ± 11.0 | 748.9 ± 31.7 | 348.5 ± 0.2 | 27.4 ± 0.3 |
| E54/A57           | 45.8 ± 0.7        | 317.5 ± 12.2 | 817.7 ± 35.0 | 350.5 ± 0.2 | 28.8 ± 0.3 |
| E54/L57           | 56.9 ± 0.6        | 356.3 ± 19.8 | 907.2 ± 55.2 | 350.0 ± 0.2 | 36.1 ± 0.4 |
| A54/A57           | 40.9 ± 0.5        | 366.0 ± 15.9 | 952.1 ± 45.0 | 351.9 ± 0.2 | 30.0 ± 0.3 |
| A54/L57           | 53.2 ± 0.9        | 395.8 ± 16.2 | 1012.5 ± 45.5 | 359.6 ± 0.2 | 38.4 ± 0.4 |
| Q54/A57           | 41.3 ± 0.5        | 316.6 ± 14.0 | 816.7 ± 39.9 | 349.8 ± 0.2 | 28.2 ± 0.3 |
| Q54/L57           | 52.9 ± 0.7        | 400.7 ± 17.6 | 1034.4 ± 49.4 | 357.0 ± 0.2 | 35.6 ± 0.4 |
Fig. 6, a and b shows the results of different DMCs for the two conserved salt bridges (Glu-36/Lys-68 and Glu-54/Arg-57) in protein Ssh10b at 298 K. For Glu-36/Lys-68, the four coupling free energy values at 298 K were 5.4, 5.9, 6.3, and 5.9 kJ/mol, and for Glu-54/Arg-57, the coupling free energy values of the four DMCs at 298 K were 2.0, 2.6, 2.1, and 2.1 kJ/mol. We can assign favorable Gibbs free energy of 5.9 ± 0.6 kJ/mol and 2.2 ± 0.4 kJ/mol to the salt bridges Glu-36/Lys-68 and Glu-54/Arg-57, respectively at 298 K.

Gibbs free energy of 6.0 ± 0.2 kJ/mol and 2.4 ± 0.4 kJ/mol to the salt bridges Glu-36/Lys-68 and Glu-54/Arg-57, respectively at 298 K.

Fig. 6, c and d shows the results of different DMCs for the two conserved salt bridges in protein Ssh10b at 353 K. For Glu-36/Lys-68, the four coupling free energy values at 353 K were 5.4, 5.9, 6.3, and 5.9 kJ/mol, and for Glu-54/Arg-57, the coupling free energy values of the four DMCs at 353 K were 2.0, 2.6, 2.1, and 2.1 kJ/mol. We can assign favorable Gibbs free energy of 5.9 ± 0.6 kJ/mol and 2.2 ± 0.4 kJ/mol to the salt bridges Glu-36/Lys-68 and Glu-54/Arg-57, respectively at 353 K. Remarkably, these results were highly consistent with those at 298 K.

**DISCUSSION**

Experimental estimates of the energy contribution of a salt bridge to protein stability have led to conflicting conclusions, ranging from them having stabilizing, insignificant, or even destabilizing effects (17–23). In some reports, electrostatic interactions of salt bridges were measured by mutating a charged residue to a non-charged residue and measuring the impact of this change on protein stability. This type of approach is unsuitable as such mutations not only remove charge-charge interactions but also alter a number of other interactions, including the desolvation penalty and the background interactions of the residue within the protein. However, DMC analysis provides an elegant method to estimate the strength of a salt bridge.

The contribution of a salt bridge (ΔΔG_{br}) to protein stability involving two charged residues can be represented as Equation 14,

\[ ΔΔG_{br} = ΔΔG_{dir} + ΔΔG_{desol} + ΔΔG_{backg} \]  

(Eq. 14)

where ΔΔG_{dir} is the direct contribution of the salt bridge to protein stability. In most situations, it is the direct charge-charge interaction, but it sometimes also includes other direct interactions, e.g. van der Waals interactions between atoms of
the two charged residues. The other two components are all indirect contributions of the salt bridges: \( \Delta \Delta G_{\text{desol}} \) is the desolvation penalty of the two charged residues, and \( \Delta \Delta G_{\text{backg}} \) is the stability contributions due to background interactions of the two charged residues with other components of the protein. Generally, the desolvation penalty is regarded as the dominant component of the indirect contributions (44).

DMC analysis is designed to cancel all interactions except the direct interactions between the two residues involved in the salt bridge. However, the validity of the method is subject to the following two assumptions. First, all indirect interactions are simply additive in nature from the single to the double mutation. Secondly, there is no interaction between the two residues in the doubly substituted mutant. Then, in an ideal DMC, according to Equations 12 and 13, indirect contributions are first removed for each residue (\(- \Delta \Delta G_{\text{mut1}} - \Delta \Delta G_{\text{mut2}}\)) and then added back again by (\(+ \Delta \Delta G_{\text{back}}\)). Hence, in an ideal DMC, the coupling free energy is the direct contribution of the salt bridge.

To test the above assumptions, some DMC analyses have been carried out alongside structural studies (34, 35). Another effective method is to construct several independent DMCs for a specific salt bridge. If the coupling free energy is independent of different DMCs, the assumptions described above are likely to be correct (36, 44).

Here, we used the DMC approach to estimate the net strength of the two highly conserved salt bridges, Glu-54/Arg-57 and Glu-36/Lys-68 in protein Ssh10b. Four independent DMCs were generated for each salt bridge to test the feasibility of this method. The coupling free energies were 2.4 \( \pm \) 0.4 kJ/mol at 298 K and 2.2 \( \pm \) 0.4 kJ/mol at 353 K for Glu-54/Arg-57, and 6.0 \( \pm \) 0.2 kJ/mol at 298 K and 5.9 \( \pm \) 0.6 kJ/mol at 353 K for Glu-36/Lys-68. Our results demonstrate that, though the stability free energy of Ssh10b decrease greatly with increasing temperature, the direct stability contribution of these two salt bridges remain almost constant, providing evidence supporting the theoretical prediction that salt bridge interactions are extremely resilient to temperature increases and thus are specially suited to promoting protein stability at high temperatures (26). This may be the reason why thermophilic and hyperthermophilic proteins have increasing numbers of salt bridges with increasing growth temperature.

It has been shown that a higher transition temperature in a hyperthermophilic protein can be obtained in three theoretical ways: 1) by shifting the stability curve to increase the overall unfolding free energy at any temperature, 2) by decreasing the \( \Delta C_p \) between the folded and unfolded states to flatten the stability curve, or 3) by shifting the stability curve toward higher temperatures (45, 46). The Ssh10b structure is stabilized by eight isolated salt bridges and two ion-pair networks (each involves three salt bridges). Because salt bridge interactions are extremely resilient to temperature increases, increasing the number of salt bridges in Ssh10b should decrease \( \Delta C_p \) between the folded and unfolded states of the protein and thus flatten its stability curve.

According to a previous report, Glu-54/Arg-57 and Glu-36/Lys-68 form two salt bridges, which are located on the surface of the Ssh10b molecule (30). However according to solvent accessible surface area (ASA) calculations, the four residues involved in these salt bridges are only partially solvent exposed. The solvent ASA of side chains of these residues are: Glu-54: 5%, Arg-57: 28%, Glu-36: 40%, and Lys-68: 58%. The two residues of the Glu-54/Arg-57 salt bridge are both located in the second \( \alpha \)-helix, while those of the Glu-36/Lys-68 salt bridge are far apart in primary sequence and located in two separate \( \beta \)-strands (Fig. 1). Because the dielectric constant inside a protein is lower than that of the protein surface, it is generally accepted that the direct stability contribution of a salt bridge buried in a protein should be higher than that of a salt bridge located on the surface (47). However, though the residues involved in the Glu-36/Lys-68 salt bridge are more exposed than those of the Glu-54/Arg-57 salt bridge, the coupling free energies of Glu-36/Lys-68 (6.0 kJ/mol at 298 K and 5.9 kJ/mol at 353 K) are much higher than those of Glu-54/Arg-57 (2.4 kJ/mol at 298 K and 2.2 kJ/mol at 353 K).

The contribution of a salt bridge to protein stability is defined as the free energy of unfolding the protein containing the salt bridge minus that of unfolding the protein without the salt bridge (26). If a salt bridge interaction could exist in both a native and unfolded state, the stability contribution of this salt bridge would be lower than that if the salt bridge only existed in the native state. In the primary sequence of Ssh10b, the Glu-54 and Arg-57 residues are separated by two hydrophobic resi-

### TABLE 2

| Salt bridge | \( \Delta \Delta G_{\text{coupl}} \) kJ mol\(^{-1} \) | Intrahelix (yes/no) |
|------------|-----------------|------------------|
| Barnase (32,47–49) | D8/R110 | 3.5 | No |
| D12/R110 | 1.4 | No |
| D12/R16 | 0.9 | Yes |
| E28/K32 | 0.3 | Yes |
| R69/D93 | 14.3 | No |
| Rubredoxin (50) | K6/E49 | 1.2 | No |
| Nt/E14 | 6.3 | No |
| Zinc finger (51) | K3/E14 | 0.8 | No |
| GCN4-p1 (52) | K8/E11 | 1.2 | Yes |
| E11/K15 | 2.1 | Yes |
| NTL9 (35) | Nt/D23 | 7.1 | No |
| Ssh10b | E36/K68 | 6.0 | No |
| E54/R57 | 2.4 | Yes |
| Protein G (53,54) | R6/E53(A44) | 2.5 | No |
| R6/E53(R44) | 2.7 | No |
| E6/R53 | 3.1 | No |
| K6/E53 | 1.6 | No |
| R44/E53(I6) | 2.2 | No |
| R44/E53(R6) | 2.4 | No |
| A repressor (17) | D14/R17 | 3.4 | Yes |
| T4 lysozyme (55) | D116/R119 | 0.4 | Yes |
| Ubiquitin (36) | E34/K11 | 3.6 | No |
| OmpA (56) | E52/K82 | 14.7 | No |
| E52/R138 | 23.5 | No |
| K68/E128 | 7.1 | No |
| E128/R138 | 2.5 | No |
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dues, Val-55 and Ile-56. In the unfolded state, the most favorable conformational shape arises when Glu-54 and Arg-57 interact with water, while residues Val-55 and Ile-56 bind to hydrophobic areas of the protein. This conformation would increase the frequency of salt bridge contacts formed by Glu-54 and Arg-57. However, the situation for the Glu-36/Lys-68 salt bridge is different: the two residues are far apart in the primary sequence, so the frequency of salt bridge contacts formed by Glu-36 and Lys-68 in the unfolded state is much lower.

To determine whether conclusions reached from our experiments on salt bridges in Ssh10b are applicable to other proteins, we analyzed published DMC experimental data for the contribution of salt bridges to protein stability in other proteins (Table 2). This confirmed that the average coupling free energy of intrahelical salt bridges (typically formed by two very close charged residues) is lower than that of salt bridges formed between residues far apart in sequence. Five of the proteins included in Table 2 have intrahelical salt bridges; coupling free energies ($\Delta \Delta G_{\text{coupl}}$) for these salt bridges determined by DMC experiments are: 0.3 and 0.9 kJ/mol for Asp-12/Arg-16 and Glu-28/Lys-32 respectively in Barnase, 3.4 kJ/mol for Asp-14/Arg-17 in a Repressor, 0.4 kJ/mol for Asp-116/Arg-119 in T4 lysozyme, 1.2 and 2.1 kJ/mol for Lys-8/Glu-11 and Glu-11/Lys-15, respectively, in GCN4-p1, and 2.4 kJ/mol for Glu-54/Lys-57 in lysozyme, 1.2 and 2.1 kJ/mol for Lys-8/Glu-11 and Glu-11/Lys-15.

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This finding provides further evidence that the average coupling free energy of intrahelical salt bridges is lower than that of salt bridges formed between residues far apart in sequence. Five of the proteins included in Table 2 have intrahelical salt bridges; coupling free energies ($\Delta \Delta G_{\text{coupl}}$) for these salt bridges determined by DMC experiments are: 0.3 and 0.9 kJ/mol for Asp-12/Arg-16 and Glu-28/Lys-32 respectively in Barnase, 3.4 kJ/mol for Asp-14/Arg-17 in a Repressor, 0.4 kJ/mol for Asp-116/Arg-119 in T4 lysozyme, 1.2 and 2.1 kJ/mol for Lys-8/Glu-11 and Glu-11/Lys-15, respectively, in GCN4-p1, and 2.4 kJ/mol for Glu-54/Lys-57 in lysozyme, 1.2 and 2.1 kJ/mol for Lys-8/Glu-11 and Glu-11/Lys-15.

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