**Abstract:** Salt bridges are very common in proteins. But what drives the formation of protein salt bridges is not clear. In this work, we determined the strength of four salt bridges in the protein GB3 by measuring the $\Delta pK_a$ values of the basic residues that constitute the salt bridges with a highly accurate NMR titration method at different temperatures. The results show that the $\Delta pK_a$ values increase with temperature, thus indicating that the salt bridges are stronger at higher temperatures. Fitting of $\Delta pK_a$ values to the van't Hoff equation yields positive $\Delta H$ and $\Delta S$ values, thus indicating that entropy drives salt-bridge formation. Molecular dynamics simulations show that the protein and solvent make opposite contributions to $\Delta H$ and $\Delta S$. Specifically, the enthalpic gain contributed from the protein is more than offset by the enthalpic loss contributed from the solvent, whereas the entropic gain originates from the desolvation effect.

Electrostatic interactions are ubiquitous in biomolecules. They play an important role in the binding of proteins to small ligands, proteins, DNA, and other molecules. It has also been shown that protein thermostability can be improved through optimizing the surface charge–charge interactions. As two ionizable residues with opposite charges approach to each other, a salt bridge is formed. But one fundamental question is what drives the formation of a protein salt bridge? For two isolated opposite charges in the gas phase, the Coulomb attraction pulls the two charges close to each other, so that the main driving force is enthalpy. However in water, free-ion associations are usually driven by entropy. For charges in a protein, the situation is more complicated because when the salt bridge is formed, both the solvent and the protein surroundings are reorganized. It is unclear how the enthalpy or entropy change upon the formation of a protein salt bridge.

In this work, we attempted to measure the enthalpy ($\Delta H$) and entropy ($\Delta S$) changes for salt bridges in the third immunoglobulin-binding domain of protein G (GB3), a 56-residue protein. The X-ray structure suggests that there are four salt bridges: K4–E15, K28–E24, K31–E27, and K50–D47 (Figure S1 in the Supporting Information). The salt-bridge formation free energy $\Delta G$ can be quantified by $\Delta pK_a$[11] that is, the $pK_a$ difference between the constituent ionizable residues in the absence and presence of the salt bridge. To obtain $\Delta H$ and $\Delta S$, it is critical to determine $\Delta pK_a$ with high accuracy at different temperatures. To achieve this goal, we first mutated the salt-bridge basic residue lysine to a histidine (e.g., K31H in the K31–E27 salt bridge), which allowed us to determine the $pK_a$ of the basic residue through pH titration (because the protein is unstable at high pH values ($pH > 11$), the $pK_a$ of lysine cannot be determined accurately through titration). Then, another conservative mutation was introduced to eliminate the salt bridge (e.g., K31H,E27Q). The free energy of salt-bridge formation $\Delta G = -2.303 kT \Delta pK_a$[11] where $R$ is the gas constant, $T$ is temperature, and $\Delta pK_a$ which corresponds to the H31 pK_a difference of two mutants, is defined by $\Delta pK_a(H31) = pK_a(K31H) - pK_a(K31H,E27Q)$. $pK_a$ values can be determined through pH titrations. In this work, the K31H mutant was $^{15}$N-labeled whereas the K31H,E27Q mutant was $^{15}$N-labeled only. The two protein samples were mixed together in a 2 mM NaCl solution and titrated with HCl or NaOH to change the pH values, and the $^1H$-$^{15}$N spectra were recorded at 283, 290.5, 298, 305.5, and 313 K with an interleaved pulse sequence to separate the signals from the two proteins (Figures 1A and Figure S2). Because there are no other histidine residues in GB3, the backbone $^1H$-$^{13}$C spectra can be used for $pK_a$ fitting of the mutated histidine residue. These spectra have a much higher signal-to-noise ratio than the side-chain spectra usually recorded for $pK_a$ fitting. Since the two proteins were dissolved in the same solution, the main $\Delta pK_a$ uncertainty originated from the pH measurement error was eliminated. The identical environment experienced by the two proteins also minimized effects caused by variation in ionic strength and temperature in otherwise separate samples.

The $pK_a$ values were determined by fitting the H31 $^{15}$N chemical shifts at different pH values to the well-known Henderson–Hasselbalch equation (Figure 1B–F and Table S1). The corresponding $\Delta pK_a$ values are shown in Figure 2. The increase in $\Delta pK_a$ with temperature indicates that the H31–E27 salt bridge is stronger at higher temperature. The same analyses were performed for the H4–E15, H28–E24, and H50–D47 salt bridges (Figures S3). The $\Delta pK_a$ values of the histidines in these three salt bridges also increase with temperature (Figure 2). Fitting of the $\Delta pK_a$ values at different temperatures to the van’t Hoff equation yielded $\Delta H$ and $\Delta S$ values for the salt bridges (Table 1). All four salt bridges have positive $\Delta H$ and $\Delta S$ values. The positive $\Delta H$
That is to say, entropy but not enthalpy drives the formation of the salt bridges. It also appears that the stronger salt bridges tend to have a more positive $\Delta H$ and $\Delta S$ (Table 1). It is not clear whether this is a general property of all salt bridges.

To further understand the enthalpy–entropy compensation in the protein salt bridge formation, molecular dynamics (MD) simulation free energy calculations were performed for GB3 in water to predict the $\Delta G$ values of two salt bridges H28–E24 and H31–E27 in the GB3 $\alpha$-helix at different temperatures by moving the negatively charged glutamate (E24 or E27) far away from its salt bridge partner (See Methods and Materials in the Supporting Information for more details). The MD results capture the trend that the salt bridges are stronger at higher temperature (Figure 3A and Figure S4). Then, $\Delta G$ was decomposed to two terms, $\Delta G_{\text{solv}}$ and $\Delta G_{\text{prot}}$, which are the contributions from the solvent and the protein, respectively.$^{[13]}$ Compared to $\Delta G$, the absolute values of $\Delta G_{\text{solv}}$ and $\Delta G_{\text{prot}}$ are much larger but with opposite signs, thus suggesting that the two components tend to cancel each other out (Figure 3A and Figure S4). $\Delta G_{\text{solv}}$ and $\Delta G_{\text{prot}}$ also show opposite behavior, that is, $\Delta G_{\text{prot}}$ decreases but $\Delta G_{\text{solv}}$ increases with temperature. The total $\Delta G$ decreases with temperature but with a much shallower slope than that of

indicates that the enthalpic change upon the salt bridge formation is unfavorable. This is a bit surprising since the electrostatic attraction between the two charges should yield an enthalpic gain. On the other hand, the positive $\Delta S$ value indicates that entropy increases as the salt bridge is formed.

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\[ \Delta G_{\text{olv}} \] The same trend for \( \Delta G \) and \( \Delta G_{\text{olv}} \) indicates that the stronger salt bridge at higher temperature is due to the contribution from the solvent instead of the protein. This is essentially a desolvation effect, that is, the charges are desolvated when they pair with each other. The importance of desolvation effect in protein charge–charge associations has been highlighted in recent computational studies.\(^{[14]}\)

To gain further insight into the salt-bridge formation mechanism, the three computational \( \Delta Gs \) were further decomposed. After converting \( \Delta G \), \( \Delta G_{\text{olv}} \), and \( \Delta G_{\text{prot}} \) into corresponding \( \Delta P_k \) values, we were able to obtain the change in enthalpy (\( \Delta H \), \( \Delta H_{\text{olv}} \) and \( \Delta H_{\text{prot}} \)) and entropy (\( \Delta S \), \( \Delta S_{\text{olv}} \) and \( \Delta S_{\text{prot}} \)) for the salt bridges through fitting the \( \Delta P_k \) values to the van’t Hoff equation (Figures 3B and Table S2).

Taking H31–E27 as an example, the \( \Delta H_{\text{olv}} \) value of \(-34.4 \pm 3.4 \) kcal mol\(^{-1}\) suggests that the protein enthalpic contribution (including the salt-bridge charge–charge attraction and the reorganization of protein residues) favors H31–E27 salt-bridge formation. In contrast, the \( \Delta H_{\text{prot}} \) value of 38.0 \( \pm 3.9 \) kcal mol\(^{-1}\) implies that the desolvation enthalpy disfavors salt-bridge formation. The total enthalpy \( \Delta H \) of 3.6 \( \pm 0.5 \) kcal mol\(^{-1}\) indicates that the overall enthalpy is unfavorable, which is consistent with the experimental \( \Delta H \) of 2.7 \( \pm 0.1 \) kcal mol\(^{-1}\).

From entropic point of view, \( \Delta S_{\text{prot}} \) of \(-32.4 \pm 8.5 \) cal mol\(^{-1}\) K suggests that the protein entropic contribution is also unfavorable, which is not surprising because salt-bridge formation restrains certain protein degrees of freedom (e.g., the side-chain dihedral of rotation of H31 and E27). On the other hand, the solvent entropic contribution favors salt-bridge formation because \( \Delta S_{\text{olv}} \) is 45.6 \( \pm 10.2 \) cal mol\(^{-1}\) K. The predicted total positive \( \Delta S \) of 13.2 \( \pm 1.7 \) cal mol\(^{-1}\) K, which is comparable to the experimental value (Table 1), indicates that the overall entropic change is favorable and that salt-bridge formation is driven by the desolvation entropy. The same conclusion can be drawn for the H28–E24 salt bridge (Table S2). It is important to have converged computational results. Based on the error estimation from the block average, the calculated thermodynamic parameters have a percentage error of 10–30% (Table S2). The computational data have reasonable accuracy and provide insight that is not gained from the experimental data alone. Owing to the charge–solvent electrostatic interaction, water molecules surrounding the charged residues are restrained. As the two oppositely charged residues move close to each other, the total charge–solvent interaction becomes weaker, which yields positive \( \Delta H_{\text{olv}} \) values, and these restrained water molecules are liberated, which increases the solvent entropy.\(^{[10]}\)

In summary, all four salt bridges in GB3 display positive \( \Delta H \) and \( \Delta S \) values, thus indicating that entropy drives the formation of salt bridges. MD simulations suggest that the solvent water and the protein make opposite contributions to \( \Delta H \) and \( \Delta S \). The main entropic gain is from the desolvation effect, that is, the release of restrained water molecules as the salt bridge is formed. It is worth mentioning that the salt bridges characterized here with histidine as the constituent basic residue are different from those in the native protein, which has lysine as the basic residue. Due to the fact that histidine is typically neutral at pH 7, the percentage of histidine residues that form salt bridges is considerably lower than that for lysine or arginine.\(^{[14]}\) But the mechanism that governs the formation of different salt bridges should be very similar.

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**Conflict of interest**

The authors declare no conflict of interest.

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