Entropy Drives the Formation of Salt Bridges in the Protein GB3

Ning Zhang⁺, Yefei Wang⁺, Liaoyuan An, Xiangfei Song, Qingshan Huang, Zhijun Liu, and Lishan Yao*

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 ΔpK_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 ΔpK_a values increase with temperature, thus indicating that the salt bridges are stronger at higher temperatures. Fitting of ΔpK_a values to the van't Hoff equation yields positive ΔH and ΔS values, thus indicating that entropy drives salt-bridge formation. Molecular dynamics simulations show that the protein and solvent make opposite contributions to ΔH and ΔS . 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.^[1] They play an important role in the binding of proteins to small ligands,^[2] proteins,^[3] DNA,^[4] and other molecules.^[5] It has also been shown that protein thermostability can be improved through optimizing the surface charge–charge interactions.^[6] As two ionizable residues with opposite charges approach to each other, a salt bridge is formed.^[7] 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.^[8] 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.^[9] 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 (ΔH) and entropy (ΔS) 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).^[10] The salt-bridge-formation free energy ΔG can be quantified by ΔpK_a ,^[11] that is, the pK_a difference between the constituent ionizable residues in the absence and presence of the salt bridge. To obtain ΔH and ΔS , it is critical to determine ΔpK_a with very 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 $\Delta G = -2.303RT\Delta pK_a$,^[11a] where R is the gas constant, T is temperature, and ΔpK_a , which corresponds to the H31 pK_a difference of two mutants, is defined by $\Delta pK_a(\text{H31}) = pK_a(\text{K31H}) - pK_a(\text{K31H, E27Q})$. pK_a values can be determined through pH titrations. In this work, the K31H mutant was ¹⁵N/¹³C-labeled whereas the K31H, E27Q mutant was ¹⁵N-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-¹⁵N 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 1 A and Figure S2).^[12] Because there are no other histidine residues in GB3, the backbone ¹H-¹⁵N 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 ΔpK_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 ¹⁵N chemical shifts at different pH values to the well-known Henderson–Hasselbalch equation (Figure 1 B–F and Table S1). The corresponding ΔpK_a values are shown in Figure 2. The increase in ΔpK_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 ΔpK_a values of the histidines in these three salt bridges also increase with temperature (Figure 2). Fitting of the ΔpK_a values at different temperatures to the van't Hoff equation yielded ΔH and ΔS values for the salt bridges (Table 1). All four salt bridges have positive ΔH and ΔS values. The positive ΔH

[*] N. Zhang,^[+] Y. Wang,^[+] L. An, X. Song, Q. Huang, Prof. L. Yao
Shandong Provincial Key Laboratory of Synthetic Biology
Qingdao Institute of Bioenergy and Bioprocess Technology
University of Chinese Academy of Sciences
Songling Road 189, Qingdao, 266061 (China)
E-mail: yaols@qibebt.ac.cn

Z. Liu

Department National Center for Protein Science Shanghai, Shanghai
Institutes for Biological Sciences, Chinese Academy of Sciences
Haik Road 333, Shanghai, 201210 (China)

[†] These authors contributed equally to this work.

Supporting information (including experimental details) and the
ORCID identification number(s) for the author(s) of this article can
be found under:
<https://doi.org/10.1002/anie.201702968>.

Figure 1. pH titration of the mixed K31H ($^{15}\text{N}/^{13}\text{C}$ -labeled, red) and K31H,E27Q (^{15}N -labeled, black) sample. A) The ^1H - ^{15}N signals (H31 at 298 K, pH 5.1) of the mixed proteins were separated by an interleaved experiment using a ^{13}C filter (Figure S2).^[12] The ^{15}N chemical shift of H31 at different pH values was fitted to the Henderson–Hasselbalch equation to yield pK_a values at 283 K (B), 290.5 K (C), 298 K (D), 305.5 K (E), and 313 K (F).

Figure 2. Fitting of the ΔpK_a values of histidines at different temperatures to the van't Hoff equation: $-2.303\Delta\text{pK}_a = \Delta\text{H}/RT - \Delta\text{S}/R$. A) ΔpK_a of H4 in the H4–E15 salt bridge (B) ΔpK_a of H28 in the H28–E24 salt bridge. C) ΔpK_a of H31 in the H31–E27 salt bridge. (D) ΔpK_a of H50 in the H50–D47 salt bridge. The fitted ΔH and ΔS values are listed in Table 1.

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 ΔS value indicates that entropy increases as the salt bridge is formed.

Table 1: Thermodynamic parameters ΔG (kcal mol^{-1}), ΔH (kcal mol^{-1}), and ΔS ($\text{cal mol}^{-1}\text{K}^{-1}$) for the four salt bridges in GB3.

	H4–E15	H28–E24	H31–E27	H50–D47
$\Delta\text{G}^{[a]}$	-0.77 ± 0.07	-0.46 ± 0.03	-1.17 ± 0.02	-0.50 ± 0.05
ΔH	0.9 ± 0.1	0.9 ± 0.1	2.7 ± 0.1	1.7 ± 0.1
ΔS	5.7 ± 0.2	4.5 ± 0.3	13.0 ± 0.3	7.5 ± 0.4

[a] ΔG is the salt-bridge-formation free energy at 298 K.

That is to say, entropy but not enthalpy drives the formation of the salt bridges. It also appears that the stronger salt bridges tends to have a more positive ΔH and ΔS (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 ΔG values of two salt bridges H28–E24 and H31–E27 in the GB3 α -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 3 A and Figure S4). Then, ΔG was decomposed to two terms, $\Delta\text{G}_{\text{solv}}$ and $\Delta\text{G}_{\text{prot}}$, which are the contributions from the solvent and the protein, respectively.^[13] Compared to ΔG , the absolute values of $\Delta\text{G}_{\text{solv}}$ and $\Delta\text{G}_{\text{prot}}$ are much larger but with opposite signs, thus suggesting that the two components tend to cancel each other out (Figure 3 A and Figure S4). $\Delta\text{G}_{\text{solv}}$ and $\Delta\text{G}_{\text{prot}}$ also show opposite behavior, that is, $\Delta\text{G}_{\text{solv}}$ decreases but $\Delta\text{G}_{\text{prot}}$ increases with temperature. The total ΔG decreases with temperature but with a much shallower slope than that of

Figure 3. Free energy of formation ΔG for the H31–E27 salt bridge predicted by MD simulations. A) The total free energy ΔG (black square), the solvent contribution $\Delta\text{G}_{\text{solv}}$ (red dot), and the protein contribution $\Delta\text{G}_{\text{prot}}$ (blue triangle) are plotted against temperature. $\Delta\text{G}_{\text{solv}}$ and $\Delta\text{G}_{\text{prot}}$ have opposite signs and largely cancel each other out. The total free energy $\Delta\text{G} = \Delta\text{G}_{\text{solv}} + \Delta\text{G}_{\text{prot}}$ becomes more negative (a stronger salt bridge) at higher temperature. B) Decomposition of ΔG , $\Delta\text{G}_{\text{solv}}$ and $\Delta\text{G}_{\text{prot}}$ to the corresponding enthalpic and entropic terms. The positive ΔS and $\Delta\text{S}_{\text{solv}}$ suggest that the driving entropy for salt-bridge formation is from the desolvation effect. All the thermodynamic parameters are also listed in Table S2.

ΔG_{solv} The same trend for ΔG and ΔG_{solv} 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 ΔG s were further decomposed. After converting ΔG , ΔG_{solv} , and ΔG_{prot} into corresponding ΔpK_a values, we were able to obtain the change in enthalpy (ΔH , ΔH_{solv} , and ΔH_{prot}) and entropy (ΔS , ΔS_{solv} , and ΔS_{prot}) for the salt bridges through fitting the ΔpK_a values to the van't Hoff equation (Figures 3B and Table S2). Taking H31–E27 as an example, the ΔH_{prot} value of $-34.4 \pm 3.4 \text{ kcal mol}^{-1}$ 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 ΔH_{solv} value of $38.0 \pm 3.9 \text{ kcal mol}^{-1}$ implies that the desolvation enthalpy disfavors salt-bridge formation. The total enthalpy ΔH of $3.6 \pm 0.5 \text{ kcal mol}^{-1}$ indicates that the overall enthalpy is unfavorable, which is consistent with the experimental ΔH of $2.7 \pm 0.1 \text{ kcal mol}^{-1}$. From entropic point of view, ΔS_{prot} of $-32.4 \pm 8.5 \text{ cal mol}^{-1} \text{ K}$ 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 ΔS_{solv} is $45.6 \pm 10.2 \text{ cal mol}^{-1} \text{ K}$. The predicted total positive ΔS of $13.2 \pm 1.7 \text{ cal mol}^{-1} \text{ K}$, 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 ΔH_{solv} values, and these restrained water molecules are liberated, which increases the solvent entropy.^[8a]

In summary, all four salt bridges in GB3 display positive ΔH and ΔS 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 ΔH and ΔS . 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.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant nos. 31661143036 to L.Y., 31600051 to Y.W., 21376254 to Q.H., and 31600690 to L.A.). A portion of this work was performed on the Bruker 600 MHz spectrometer at the National Center for Protein Science Shanghai, CAS, China.

Conflict of interest

The authors declare no conflict of interest.

Keywords: enthalpy · entropy · NMR spectroscopy · protein structures · salt bridges

How to cite: *Angew. Chem. Int. Ed.* **2017**, *56*, 7601–7604
Angew. Chem. **2017**, *129*, 7709–7712

- [1] B. Honig, A. Nicholls, *Science* **1995**, *268*, 1144–1149.
- [2] a) G. J. Rocklin, S. E. Boyce, M. Fischer, I. Fish, D. L. Mobley, B. K. Shoichet, K. A. Dill, *J. Mol. Biol.* **2013**, *425*, 4569–4583; b) E. Persch, O. Dumele, F. Diederich, *Angew. Chem. Int. Ed.* **2015**, *54*, 3290–3327; *Angew. Chem.* **2015**, *127*, 3341–3382.
- [3] a) C. Kiel, L. Serrano, C. Herrmann, *J. Mol. Biol.* **2004**, *340*, 1039–1058; b) N. A. Lakomek, A. Draycheva, T. Bornemann, W. Wintermeyer, *Angew. Chem. Int. Ed.* **2016**, *55*, 9544–9547; *Angew. Chem.* **2016**, *128*, 9696–9699.
- [4] R. Rohs, S. M. West, A. Sosinsky, P. Liu, R. S. Mann, B. Honig, *Nature* **2009**, *461*, 1248–U1281.
- [5] A. M. Knight, P. H. Culviner, N. Kurt-Yilmaz, T. S. Zou, S. B. Ozkan, S. Cavagnero, *ACS Chem. Biol.* **2013**, *8*, 1195–1204.
- [6] a) A. V. Gribenko, M. M. Patel, J. Liu, S. A. McCallum, C. Y. Wang, G. I. Makhatadze, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 2601–2606; b) F. O. Tzul, K. L. Schweiker, G. I. Makhatadze, *Proc. Natl. Acad. Sci. USA* **2015**, *112*, E259–E266; c) S. Tripathi, A. E. Garcia, G. I. Makhatadze, *J. Phys. Chem. B* **2015**, *119*, 13103–13112.
- [7] a) T. H. Walther, C. Gottselig, S. L. Grage, M. Wolf, A. V. Vargiu, M. J. Klein, S. Vollmer, S. Prock, M. Hartmann, S. Afonin, E. Stockwald, H. Heinzmann, O. V. Nolandt, W. Wenzel, P. Ruggerone, A. S. Ulrich, *Cell* **2013**, *152*, 316–326; b) C. W. Lee, H. J. Wang, J. K. Hwang, C. P. Tseng, *Plos One* **2014**, *9*, e112751.
- [8] a) Y. Marcus, G. Hefter, *Chem. Rev.* **2006**, *106*, 4585–4621; b) V. Petruskas, E. Maximowitsch, D. Matulis, *J. Phys. Chem. B* **2015**, *119*, 12164–12171; c) J. C. Fu, J. B. Schlenoff, *J. Am. Chem. Soc.* **2016**, *138*, 980–990.
- [9] a) A. Warshel, S. T. Russell, A. K. Churg, *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 4785–4789; b) C. N. Schutz, A. Warshel, *Proteins Struct. Funct. Bioinf.* **2001**, *44*, 400–417.
- [10] J. P. Derrick, D. B. Wigley, *J. Mol. Biol.* **1994**, *243*, 906–918.
- [11] a) K. K. Lee, C. A. Fitch, B. Garcia-Moreno, *Protein Sci.* **2002**, *11*, 1004–1016; b) K. L. Baran, M. S. Chiment, J. L. Schlessman, C. A. Fitch, K. J. Herbst, B. E. Garcia-Moreno, *J. Mol. Biol.* **2008**, *379*, 1045–1062.
- [12] L. Y. An, Y. F. Wang, N. Zhang, S. H. Yan, A. Bax, L. S. Yao, *J. Am. Chem. Soc.* **2014**, *136*, 12816–12819.

- [13] S. Boresch, G. Archontis, M. Karplus, *Proteins Struct. Funct. Genet.* **1994**, 20, 25–33.
- [14] a) R. Salari, L. T. Chong, *J. Phys. Chem. B* **2012**, 116, 2561–2567;
b) K. Okazaki, T. Sato, M. Takano, *J. Am. Chem. Soc.* **2012**, 134, 8918–8925.
- [15] J. N. Sarakatsannis, Y. Duan, *Proteins Struct. Funct. Bioinf.* **2005**, 60, 732–739.

Manuscript received: March 22, 2017
Revised manuscript received: April 27, 2017
Version of record online: May 19, 2017