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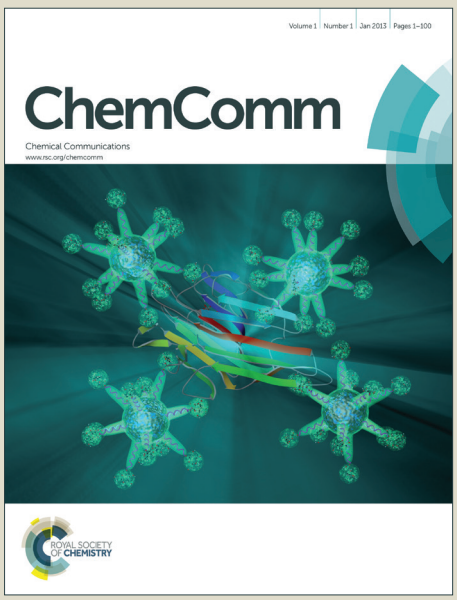

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Effect of salt on the formation of salt-bridges in β-hairpin peptides

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Salt-bridges ubiquitously form between oppositely charged moieties in proteins. Here we quantify changes in population of salt-bridged β-hairpin peptides due to added salt, and determine the thermodynamic driving forces and cooperativity of salt-bridge formation under these conditions. We find only a fraction of salt-bridged folded conformations at physiologically relevant salt concentrations.

Ranging from directing and preserving the native fold of proteins to driving intermolecular interactions, salt-bridges (SBs) that form between opposite charges in biological macromolecules play crucial roles. Nonetheless, salt-bridging in proteins has emerged as a complex and diverse process, which sensitively depends on the specifics of macromolecular interactions. In fact, many studies report minor or even destabilizing contributions of SBs to folding.[1–4] The complexity of SBs is further exacerbated by solution conditions. Specifically, cellular salt concentrations that are carefully regulated are known to perturb electrostatic interactions[5]. Yet, despite its importance, the effect of salt on SB formation has scarcely been studied.

To probe into the way salt affects SBs, we have followed the extent of SB formation in a set of model peptides. At equilibrium, these peptides (Fig. 1) reside in one of two states as determined previously by NMR and circular dichroism (CD) spectroscopy[6]: an unfolded and a folded β-hairpin ensemble. The peptides differ in their SB arrangement: the K1K2 peptide can form two SBs with a single negative charge (referred to as a salt-bridge triad), and the peptides K1 and K2 each form a single SB. Differences in charge positioning in the homologues were achieved by acetylation of the lysine side-chain (Fig. 1). The positive charges reside on the lysine side-chains near the acetylated N-terminus of the peptides, so that upon folding into a β-hairpin, these are in proximity to the free acid at the C-terminus (CT), as determined previously using NMR spectroscopy.[6] This configuration allows us to examine sequence-distant, solvent exposed SBs that are different from the relatively well studied SBs formed between neighboring residues (e.g. those in α-helices[7–9]). SB formation can be eliminated altogether by means of CT protonation at low pH. We follow the effect of NaCl on the peptide folding equilibrium by titrations with the salt. These titrations not only inform on how SBs are affected by ionic strength, but also help to delineate the fraction of salt-bridged conformations within each homologue.

Our study shows that at low salt concentrations, when a single SB can form between the β-hairpin ends, the folded ensemble has only a small extent of salt bridging that is further reduced as salt concentrations increase. Moreover, the salt-bridged K1K2 peptide population is almost exactly the sum of the two isolated single salt-bridges, indicating a non-cooperative (additive) behavior for the triad. Importantly, we show that at concentrations ranging up to those of biological relevance, added salt affects SB formation primarily through non-specific screening of electrostatic interactions, and as a result reduces the salt-bridged population in the folded ensemble.

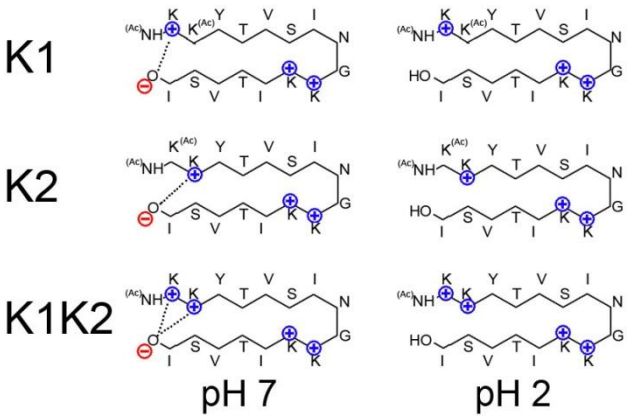

Fig.1 Three peptide homologues used in this study at pH 7 and pH 2. Charges are in blue or red. The scheme illustrates the proximity of charges upon folding.

We begin by discussing the folding free energy, ΔG, of each peptide as determined by CD. These peptides afford two major practical advantages: (i) all homologues at all solution conditions (pH, temperature, and salt concentration) share an isodichroic point in the CD spectra at $\lambda = 208 \pm 1$ nm, see SI Fig. S3-S5, and (ii) because of the relatively low folding free energy (SI Fig. S6) even small perturbations to stability are easily detectable. The isodichroic point implies that the same two ensembles make up the entire peptide population in all homologues and conditions, at least as discernible by CD. It has previously been shown that these ensembles can be assigned to either the folded, β-sheet ensemble (F), or the unfolded ensemble (U).[6,10] By resolving the spectra for the fully folded and fully unfolded ensembles (SI section 3a and Fig. S5) the folding free energy of the peptide is determined as $\Delta G = -RT \ln\left(C^{F}/C^{U}\right)$, where C^{F} and C^{U} are the molar concentrations of folded and unfolded peptide, respectively.

For all peptide homologues, Fig. 2A shows the change in ΔG due to addition of salt at concentration C_{salt},

$\Delta G_{salt} = \Delta G(C_{salt}) - \Delta G(C_{salt} = 0)$, at pH 7. Under these conditions, ΔG_{salt} changes non-monotonically for all peptides; the initial destabilization peaks, and then turns stabilizing at higher salt concentrations as seen also for larger proteins.[11]

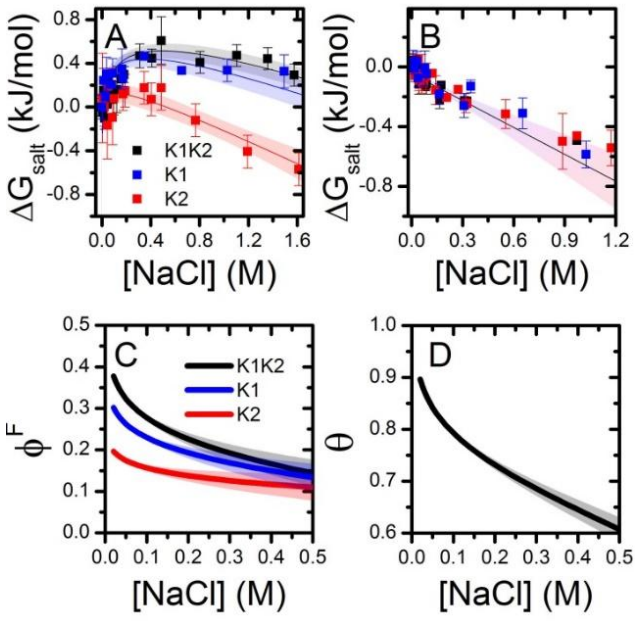

Fig.2. Salt effects on salt-bridging. Folding free energy with salt concentrations at (A) pH 7, and (B) pH 2, for the three homologues in Fig. 1. Lines are fits to Eq. 1, and bars are standard deviation of averages from at least 3 repeats. Shaded areas represent standard error of the fit. (C) Fraction of folded peptide containing SB *vs* salt concentration for all three homologues. (D) Cooperativity of SB formation in K1K2 *vs* salt concentration. Shaded areas in (C) and (D) are calculated from errors for folding equilibrium constants in (A), and propagated to estimate the error in ϕ^F and θ, respectively.

Record and co-workers have shown that this trend could be well described as a sum of electrostatic and preferential interaction terms, [5,12]

$$\Delta G_{salt} = a + b \ln C_{salt} + mC_{salt} \quad (1)$$

Here, *b* describes the magnitude of the approximate Debye-Hückle term related to screening of the electrostatic attraction by the salt ions. The prefactor *m* represents the so-called *m-value*, which describes the magnitude of the non-electrostatic (non-charge-charge) interactions, associated with the preferential exclusion of ions from peptide or protein interfaces. This interaction has been shown to be generally stabilizing (favors the F state) and is typically linear in salt concentration.[13,14] The offset *a* is a fitting parameter specific to each peptide.

The values for *a*, *b*, and *m* derived from fitting the data points shown in Fig. 2A are reported in SI Table 2. Interestingly, the *m* values are identical within error for all homologues ($m_{K1} = -450 \pm 50$, $m_{K2} = -550 \pm 90$, $m_{K1K2} = -450 \pm 50$ J/mol/mol$_{NaCl}$). This implies that the change in homologue surfaces upon folding are scarcely altered by acetylation of lysines, so that changes in preferential interactions upon folding are also the same for all homologues. This result also suggests a relatively modest preferential interaction of Na^+ and Cl^- ions with the charged moieties. Notably, the preferential interaction depends on the identity of the ions, in line with the so-called Hofmeister series. For example, we have previously shown that salts that stabilize protein more strongly than NaCl, such as $NaSO_4$, have a stronger effect on ΔG_{salt}.[12] This is particularly true at high salt concentrations (> 0.5 M), while at physiological concentration ΔG_{salt} is dominated by electrostatic interactions and are insensitive to the identity of salt.

In contrast to the non-electrostatic terms, direct electrostatic interactions are significantly different between the homologues. A higher value of *b* implies stronger electrostatic contributions to the stabilization of the folded state. Interestingly, although in both K1 and K2 homologues one SB may form, we find that $b_{K1} = 160 \pm 20$ J/mol is about twice the value of $b_{K2} = 70 \pm 30$ J/mol. This highlights how two single SBs that form with the same opposite charge can show very different degrees of stabilization, and is in line with previous investigations of salt-bridging in this peptide.[15] Moreover, we find that $b_{K1} + b_{K2} \approx b_{K1K2} = 220 \pm 20$ J/mol. This suggests that the interactions of both lysine side chains with the CT in K1K2 are additive (non-cooperative), at least at low salt concentrations.

At pH 2, the CT is protonated, effectively abolishing all possibility for salt bridging. This elimination is manifested in the linear dependence of ΔG_{salt} on salt concentration, Fig. 2B. This linearity implies that here only salt exclusion from the peptide interface contributes to peptide stability.[16,13,17] Indeed, the slope (m = -650 ± 70 J/mol/mol$_{NaCl}$) is close to the *m-values* at neutral pH (we comment on the small deviations from this value in SI section 3c). Moreover, all peptide homologues show this same slope, indicating that the preferential exclusion of salt from the F and U ensembles is the same also under acidic conditions.

Taken together, the effects of salt on ΔG indicate that the main contribution to variations in ΔG result from differences in the SBs formed between the lysines and the CT, rather than from the changes introduced by acetylation to the lysine side chains. This allows us to consider changes in ΔG_{salt} of the three homologues as directly resulting from differences in salt bridging.

We next determine, for each homologue, the effects of salt on the fraction of salt-bridged conformations within an ensemble, defined as $\phi^i = C^i_{SB} \Big/ \left(C^i_{SB} + C^i_{noSB}\right)$, where $i = F, U$. We consider four possible population concentrations C^i_j for each homologue, where *j* = SB, noSB (containing at least one SB, or no SB, respectively). The folding equilibrium at pH 7 can now be expressed as:

$$K^{pH7} = \frac{C^F_{noSB}\left(1 + \frac{C^F_{SB}}{C^F_{noSB}}\right)}{C^U_{noSB}\left(1 + \frac{C^E_{SB}}{C^U_{noSB}}\right)} = \frac{C^F_{noSB}\left(1-\phi^U\right)}{C^U_{noSB}\left(1-\phi^F\right)} \quad (2)$$

The experimentally determined absence of salt bridging at low pH implies that $K^{pH2} = C^F_{noSB} \Big/ C^U_{noSB}$. It follows that $K^{pH7} \Big/ K^{pH2} = \left(1-\phi^U\right) \Big/ \left(1-\phi^F\right)$. Finally, considering salt bridging to be extremely rare in the U state so that $\phi^U \to 0$, we derive an expression for the fraction of folded *and* salt-bridged conformations for each homologue $\phi^F \cong 1 - \left(K^{pH2} \Big/ K^{pH7}\right)$. Setting $\phi^U \to 0$ is reasonable, at least for low salt concentrations, because the CD spectra of the U ensemble has

been shown to be close to that of a typical unfolded ensemble.[6,10] Moreover, molecular dynamics simulations of the unfolded K1K2 ensemble show that less than 3% of the population has a 6 Å or shorter distance between any two opposite charges (SI section 3f and Fig. S9). This suggests that the probability of SB formation in the unfolded state is highly unlikely.[18] Note, however, that at high salt concentrations, where $\phi^{F} \to 0$ due to salt screening, the approximation $\phi^{U} \to 0$ is no longer valid. In this limit $K^{pH7} \cong K^{pH2}$, which would mean that the folding free energy of a peptide at pH 7 is the same as when no SBs exist. Conversely, when $\phi^{F} \to 1$, all folded conformations are salt-bridged, and $K^{pH7} >> K^{pH2}$, so that $K^{pH7} \to C^{F}_{SB} / C^{F}_{noSB}$. Nevertheless, the results shown in Fig. 2A clearly show a folded population that exists in the absence of salt-bridges, so that the limit of $\phi^{F} \to 1$ is not realized.

Figure 2C shows that in the absence of added salt ϕ^{F} is larger for K1 ($\phi^{F} = 0.3$) than for K2 ($\phi^{F} = 0.2$), indicating a greater tendency for the folded conformations to form SBs in K1. This is in agreement with K1 showing stronger electrostatic propensity towards salt-bridging, and highlights how different salt-bridge orientations result in different extents of salt bridging within the folded state ensemble. Interestingly, ϕ^{F} is higher for K1K2than either of the single bridged homologues. Expectedly, ϕ^{F} falls for all homologues as salt concentrations (and subsequent electrostatic screening) increase.

The fraction ϕ^{F} can be used to define the equilibrium constant of SB formation within the folded ensemble $\alpha = \phi^{F} / \left(1-\phi^{F}\right) = C^{F}_{SB} / C^{F}_{noSB}$, and the corresponding free energy $\Delta G_{\alpha} = -RT \ln \alpha$. The contributions of entropy and enthalpy to SB formation within the F ensemble in each analogue is determined from the variation of ΔG_{α} with temperature, SI Fig. S7B. This analysis reveals that for all homologues, the salt-bridging is dominated by a loss in entropy, with little or no change in enthalpy. The lack of enthalpic contributions is in line with SB formation that is driven by the release of counter-ions into solution[19]. However, the extent of ion release upon SB formation, as determined by the slope $\partial \Delta G_{\alpha} / \partial \ln C_{salt}$ (SI section 3e, and Fig. S8), is smaller than the expected full ion pair per SB. Importantly, here we are concerned with SBs that are both solvent exposed and located far apart in the peptide sequence, and hence their formation may be associated with significant entropic penalties, either from conformational restrictions within the peptide itself[20] or due to changes in the surrounding solution. Moreover, salt dependent changes in peptide conformation or solution interactions may explain the smaller than expected counter ion release (SI section 3e). Indeed, our analysis indicates different entropic losses from salt-bridging in K1 and K2, suggesting that differences in the position of the SB are sufficient to change the overall entropic cost.

The ratio θ, defined as $\theta = \alpha_{K1K2} / \left(\alpha_{K1} + \alpha_{K2}\right)$ describes the cooperativity in SB formation in the K1K2 triad. At the lowest salt concentration tested, we find $\theta \approx 0.9$, indicating near lack of cooperativity (Fig. 2D). At higher salt concentrations we find negative cooperativity. This finding is in contrast to other studies that found a synergistic effect for triads in certain proteins.[21,7,22] Notably, these reports were performed in relatively low (up to 0.05 M) salt concentrations. We find that the cooperativity in SB triads is sensitive to ambient salt, especially at and below physiological concentrations (≈ 0.15 M).

In conclusion, we have provided several new insights to salt-bridge formation. The main lesson from this analysis is the low extent of salt-bridging in the folded ensemble at physiological ionic strengths. Our analyses indicate that this is mainly a result of electrostatic screening induced by the added electrolytes. This stresses the importance of ionic strengths used for *in-vitro* experiments due to electrostatic screening. Preferential interaction effects of electrolytes on SB formation are secondary at low to physiological concentrations, but may play an important role in peptide folded state stabilization at higher salt concentrations (above ~0.5 M), as present in some halophillic organisms.[23–25]

Our results further highlight the wide variability in SB strength, as found for the different peptide analogues. Moreover, we find that, in contrast to previous reports,[7,22] a SB triad may act non-cooperatively, especially at high ionic-strengths. The temperature dependence of SB formation reveals almost no net contributions of enthalpy, while results from the addition of salt suggest that changes in entropy upon SB formation are consistent with contributions from a counter-ion release mechanism. These contributions, in turn, are likely mitigated by entropic penalties associated with reduced conformational and orientational freedom of peptide or water upon salt-bridging, that lead to the differences between peptide analogues. Finally, we find that the presence of salt acts to decrease the population of salt-bridged conformations, and that the strongest effect of salt on salt bridging is experienced at physiological ionic strength and below. This work underscores the sensitivity of salt-bridges to local changes in their solvating environment, and suggests that SBs may play crucial roles mainly in "fine-tuning" the folded conformation to thermodynamically prefer the biologically active state, as has previously been suggested.[26–28,1]

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Notes and references

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