Cooperative Helix Stabilization by Complex Arg–Glu Salt Bridges

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ABSTRACT **Among the interactions that stabilize the native state of proteins, the role of electrostatic interactions has been difficult to quantify precisely. Surface salt bridges or ion pairs between acidic and basic side chains have only a modest stabilizing effect on the stability of helical peptides or proteins: estimates are roughly 0.5 kcal/mol or less. On the other hand, theoretical arguments and the occurrence of salt bridge networks in thermophilic proteins suggest that multiple salt bridges may exert a stronger stabilizing effect. We show here that triads of charged side chains, Arg^+–Glu^-–Arg^+ spaced at *i*,*i*+4 or *i*,*i*+3 intervals in a helical peptide stabilize alpha helix by more than the additive contribution of two single salt bridges. The free energy of the triad is more than 1 kcal/mol in excess of the sum of the individual pairs, measured in low salt concentration (10 mM). The effect of spacing the three groups is severe; placing the charges at *i*,*i*+4 or *i*,*i*+3 sites has a strong effect on stability relative to single bridges; other combinations are weaker. A conservative calculation suggests that interactions of this kind between salt bridges can account for much of the stabilization of certain thermophilic proteins. Proteins 2001;44:123–132.**

INTRODUCTION

The native structure of proteins is stabilized by hydrophobic effects, electrostatic interactions, and hydrogen bonds that together counter the intrinsic entropy of the polypeptide chain.[1] While each interaction plays a role in the native state of proteins, there is ongoing debate concerning their quantitative contributions. The role of electrostatic interactions has proven particularly controversial. In 1978, Perutz[2] observed that the main difference between a thermophilic and a normal ferrodoxin was in the enhanced number of surface salt bridges, which appeared to confer additional stabilization to the thermophilic protein. The strength of salt bridges has been measured in peptide and protein models. Free energy values range from 0.5 kcal/mol or less for a surface-exposed salt bridge[3,4] to as much as 5 kcal/mol for a single salt bridge in the core of lysozyme.[5] The importance of salt bridges in protein stabilization has been questioned in recent experimental and theoretical studies. Waldburger et al.[6] replaced a cluster of salt bridges in *Arc* repressor by a set of hydrophobic residues that were more stabilizing. Theoretical studies of solvation suggest that internal or external salt bridges contribute only marginally to protein stability.[7] The high cost of desolvating ions within the interior or near the surface of a protein is thought to offset the expected gain in strength of ionic interactions in a low dielectric constant medium. Proton transfer can also occur in this condition.

The role of electrostatic interactions in stabilizing proteins is of particular interest in the case of thermophilic proteins. Several proteins from thermophilic organisms contain networks of surface salt bridges absent from their less stable analogs; the increasing number of bridges and their complexity correlate with the thermal stability.[8] Networks of bridges involve multi-centered arrays of interacting charged side chains rather than isolated pairs of oppositely charged groups. While complex salt bridges are found in proteins from all organisms,[9] those in proteins from thermophiles may involve larger sets of interacting clusters of charged side chains.

Site-directed mutagenesis has been used to delete or create potential salt bridges in proteins[10]; addition of pairs of appropriately spaced charged groups to T-4 lysozyme led to little or no stabilization of that protein. Horovitz et al.[11] introduced a cycle of amino acid substitutions to determine the strength of a potentially stabilizing salt bridge triad on the surface of barnase. They found a coupling interaction of 0.8 kcal/mol that stabilized the triad relative to the sum of isolated pairs. The resulting salt bridges, however, did not stabilize barnase by as much as 3 Ala side chains at the same sites. Salt bridge clusters have been introduced into proteins with inconsistent results. Spek et al.[12] reported that a GCN4 leucine zipper is stabilized by 1.7 kcal/mol on introducing a triad of Arg–Glu–Arg at the helix surface. In this case, the salt bridges proved more stabilizing than three Ala side chains at the same sites. However, the presence of many additional charged side chains at sites close to the mutations complicated the analysis in this case, and hence the generality of the conclusion. Addition of salt bridges to glutamate dehydrogenase according to a pattern identified in a hyperthermophilic version of the protein[13] revealed only a 0.5°C enhancement in stability, in contrast to the correlation reported by Yip et al.[8] Thus,

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there is a need for further experiments to clarify the role of multiple salt bridges on protein stabilization, and especially to clarify quantitative differences. The present study uses a highly simplified model system, with a rigorously controlled background of charged side chains.

Peptide models have played a major role in the effort to define the contribution of specific amino acid side chain interactions to stabilizing helical structure.[14–16] The main advantages of peptides are that the background can be specified precisely, the effects of weak interactions between side chains can be readily detected by CD or NMR, and quantitative free energy values can be derived by fitting CD spectral data to helix–coil transition models. Peptide models have been used to evaluate the interaction free energies between pairs of side chains in a helix: examples include salt bridges,[4] aromatic and hydrophobic interactions,[16] cation–aromatic interactions,[17] etc. Here we show that spacing Arg and Glu side chains appropriately can generate non-additive free energy effects on helix stability. Similar mechanisms might operate in other side chain–side chain interactions as well, contributing to the cooperativity of protein folding.

A host system of Ala- and Gln-containing peptides has been extensively studied to evaluate the stability of salt bridges at different positions and spacings in alpha helices.[4,18,19] The free energy of a given side chain–side chain interaction can be determined by fitting CD data to theoretical helix–coil equilibrium models (see Kallenbach et al.[15]). In this way, the free energy contributions to helix stability of ion pairs between charged amino acids have been evaluated. A single salt bridge exerts only a moderate effect on the free energy of a helix: for example, a single Glu–Lys pair spaced at positions $i,i+4$ on the surface stabilizes helix by about 0.5 kcal/mol in low salt.[3,4] Spacing and order of the participating side chains are important: strong interactions are seen at spacings of charged groups at positions $i,i+3$ and $i,i+4$, but not at $i,i+2$ or $i,i+5$.[4] Generally, arrangements in which the acidic side chain is N terminal to the basic residue are more stable than the opposite.[4,20] Addition of neutral salt has only a moderate destabilizing effect on salt bridges, reflecting the high local concentration of the pairing groups and the contribution of H-bonding.[4,18] The thrust of these studies is that solvent-exposed salt bridge pairs can stabilize helical structure and, therefore, the native state of a protein, albeit modestly.

If surface ion pairs individually are weak, it is possible that triplets and more complex arrays are stronger.[21] We have reported previously that triadic salt bridges between Arg and Glu groups at solvent-exposed sites stabilize coiled– coil helical structure in the GCN4 leucine zipper.[12] In addition, we detected a strong stabilizing interaction (1.5 Kcal/mol in 10 mM salt) between Arg and two neighboring acidic side chains, Glu and Asp, in a short prenucleated peptide fragment.[22] The strength of this interaction was determined by monitoring the dependence of NH exchange rates on pH. Since titration registers the ionic component of a salt bridge and not that from hydrogen bonding, the total stabilizing effect of this bridge is still higher.

To quantify the interactions of multiple salt bridges, a simpler model system—isolated helical peptides—can be used. These allow determination of the strength of side chain interactions, avoiding the problem of side chain interactions with other residues as occurs in many proteins. Since salt bridges have a hydrogen bonding contribution in addition to the charge interaction, one needs to evaluate these separately. In a previous study of the helix propensity of Ala, we found that introducing short charged side chains such as ornithine at the ends of peptides helped to solubilize chains with up to 13 contiguous Ala residues.[23] This makes it possible to introduce charged side chains into a helical peptide without interactions from internal H-bonding residues that have been used in other studies to solubilize molecules with high alanine content.[4,19]

We present here a study of a series of alanine-based peptides containing charged side chains at different spacings, designed to allow quantitative evaluation of the role of Arg–Glu–Arg salt bridge triads on helix stability. As control, we use a peptide with Arg and Glu side chains spaced at helical positions that prevent their interaction ($i,i+5$). This arrangement places the two side chains on non-contiguous faces of a helix as well as far apart.[24] Conservation of composition within the series of peptides avoids differences in intrinsic helix propensity among the chains: differences in helicity then reflect positional effects or side chain–side chain interactions. It also avoids differences in reference states with charged or uncharged side chains that can complicate evaluation of electrostatic effects.[7] The results show that triadic salt bridges can be more stabilizing than the component ion pairs, by more than 1 kcal/mol in overall free energy in low salt solution. The results underline the importance of salt bridges in protein stabilization and help to rationalize the frequency of occurrence of multiple salt bridges in thermophilic proteins.

MATERIALS AND METHODS

Peptide Synthesis and Purification

Peptides were synthesized by solid phase peptide synthesis on a Rainin PS3 automated synthesizer using Rink resin (Advanced Chemtech), using Fmoc chemistry as described previously.[23] Cleavage from the resin and removal of side chain protecting groups was performed with 90% TFA in the presence of the scavengers anisole and H_2O. Crude peptides were precipitated in cold ether, dissolved in water, and lyophilized. Purification was performed by HPLC on a Delta Pak C18 reverse phase semi-preparative column. The molecular weight of the peptide was confirmed by MALDI mass spectrometry using a Kratos MALDI I linear time-of-flight spectrometer.

CD Measurements

Stock solution concentrations were determined by tyrosine absorbance in 6M Guanidine HCl (ϵ_{275} = 1,450 $M^{-1}cm^{-1}$).[25] Stock solutions were prepared in 10 mM

phosphate buffer (pH = 7.0) at a concentration of 500–1,500 mM. CD measurements were performed at a peptide concentration of 50 mM in 10 mM phosphate buffer pH 7 at 4°C. Salt experiments were performed by preparing a 3 M NaCl solution in 10 mM phosphate buffer and diluting with buffer to 0.5 M, 1.0 M, and 2.5 M NaCl. pH experiments were performed by diluting a stock solution of peptide in citrate-phosphate-borate buffer[24] prepared at different pH values between pH 2 and pH 13. CD measurements were recorded on an Aviv DS 60 CD spectrometer equipped with a temperature controller. The helical content of each peptide was determined from the mean residue CD at 222 nm, $[\Theta]_{222}$ (deg cm^2 dmol^{-1}) correcting for the length of the chains according to Manning and Woody.[26] Each reported CD value is the mean of at least 6 determinations, in which both the concentration of the sample and the CD value were measured. The values are thus reliable to within ± 400 deg cm^2 dmol^{-1}. The wavelength of the instrument was calibrated by means of a (+)-10-camphorsulfonic acid standard.[27]

NMR Spectroscopy

^{1}H NMR spectra were recorded on a Varian UNITY 500 spectrometer. We used the States method[28] to obtain phase-sensitive clean-TOCSY[29,30] with a mixing time of 80 ms. NOESY experiments[31–33] were run with a mixing time of 400 ms. Water suppression was achieved using a Watergate sequence.[34] The spectral width in each dimension was 5,500 Hz. Each 2D data set contained 512 FIDs with 2k complex data points each, obtained by collecting 32 added free induction decays after 4 dummy scans. Spectra were Fourier transformed in both t2 and t1 dimensions after apodization with a shifted square sine bell function, typically with an 80° phase shift. Zero filling was done in the t1 dimension to obtain a final matrix of 2,048*1,024 real points. H–H exchange rates were measured using a modified Watergate 1D sequence with pre-saturation of the water signal for variable lengths of time (from 50 ms to 3 s). Chemical shift changes were monitored by means of 1D Watergate sequences at several temperatures (5, 10, 15, 20, 25, and 30°C). NMR data were processed using VNMR (version 6.1A). Samples were prepared by dissolving peptides in 10 mM phosphate buffer (pH = 7, 10% D_2O) to a concentration of approximately 5 mM. The sodium salt of 3-(trimethysilyl)-[3,3,2,2-^{2}H] propionic acid was used as an internal chemical shift reference.

Data Analysis

Analysis of the free energy contribution of single or multiple salt bridges from CD data was carried out using a modified Zimm–Bragg multi-state helix–coil transition model as described previously.[35,36] The relation between the helix content (f) and CD is taken as $f = -[\Theta]_{222}/34{,}000$, where −34,000 is the estimated molar residue CD signal at 222 nm ($[\Theta]_{222}$) for an α helix of 24 residues.[36]

In addition to the nucleation constant σ and a set of helix propagation constants s_i corresponding to each species of amino acid (i) in the sequence, we explicitly introduce the additional equilibrium constant $\gamma = \exp(-\Delta G_{el}/RT)$, where ΔG_{el} refers to the interaction between charged side chains. The weighting for a chain of N residues is generated recursively from the weights of shorter chains using equations described by Gans et al.[35] Inter-side chain interactions between R and E residues spaced at $i,i\pm3$ or $i,i\pm4$ are weighted by the additional stability constant γ. The nucleation constant is assumed to be independent of sequence, with a value of 0.004.[36] Intrinsic helix propensities that were used were the following: $s_{Ala} = 1.6$, $s_{Arg} = 1.1$, $s_{Glu} = 0.43$, $s_{Orn} = 0.53$.[37,38] We fit data to helix–coil transition models for isolated helical chains.[3,35]

TABLE I. The Sequences of Peptides Used in this Study

Peptide	Sequence[a]
RER5-5	Ac-OOAAAARAAAAEAAAARAAAAOOY-NH_2
RER5-4	Ac-OOAAAARAAAAEAAARAAAAOOY-NH_2
RER4-5	Ac-OOAAAAARAAAEAAAARAAAAOOY-NH_2
RER5-3	Ac-OOAAAARAAAAEAARAAAAAOOY-NH_2
RER3-5	Ac-OOAAAAAARAAEAAAARAAAAOOY-NH_2
RER4-4	Ac-OOAAAAARAAAEAAARAAAAOOY-NH_2
RER4-3	Ac-OOAAAAARAAAEAARAAAAAOOY-NH_2
RER3-4	Ac-OOAAAAAARAAEAAARAAAAOOY-NH_2
RER3-3	Ac-OOAAAAAARAAEAARAAAAAOOY-NH_2

[a]Ac = acetyl; O = ornithine; A = alanine; **R** = arginine; **E** = glutamic acid; Y = tyrosine.

RESULTS

Peptide Design

The peptides of this study were designed to provide a simple model system to evaluate the quantitative contribution to helix stability of electrostatic interactions among multiple charged residues. The series explores the ability of Arg and Glu to interact along the helix surface and allows quantitative comparison with controls that form simple pair-wise salt bridges and a chain lacking any of the putative stabilizing interactions. Each peptide is 24 residues in length with the same amino acid composition (Table I). By shifting the positions of two arginines with respect to a central glutamate residue, so as to occupy spacings of $i,i\pm3$ and $i,i\pm4$ on either side (RER4-4; RER3-3; RER3-4; and RER4-4), we assess the ability of three charged residues to interact over a length of up to 8 residues. Spacing one Arg at $i,i\pm3$ or $i,i\pm4$ positions from the central Glu while fixing the second Arg at $i,i\pm5$ (RER3-5; RER4-5; RER5-4; RER5-3) allows us to determine the stabilizing effect of single Glu–Arg salt bridge pairs. The peptide RER5-5 with two Arg residues at a spacing of $i,i\pm5$ with respect to the central Glu minimizes interactions among the internal charged residues in α-helical structure and serves as reference for the series. Maintaining the Glu side chain at the middle of each chain reduces the anticipated effects of helix dipole interactions, although they cannot be eliminated completely because the helix distribution within most peptides is asymmetric.[41]

Alanine was selected as the major component in these peptides because of its high propensity to form helix in

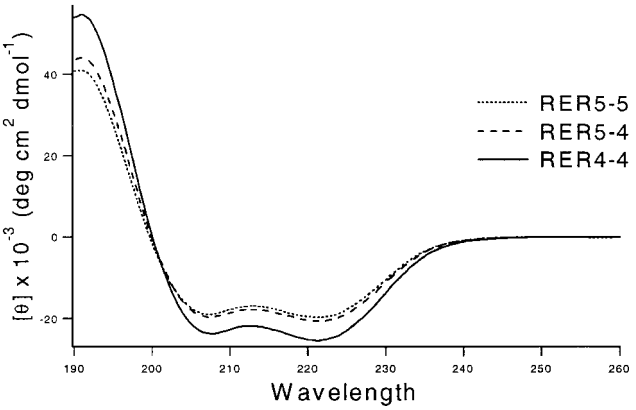

Fig. 1. Circular dichroism spectra of RER5-5, RER5-4, and RER4-4 in 10 mM phosphate buffer pH 7 at 4°C. The peptide concentrations are 50 mM in each case as determined by tyrosine absorbance at 275 nm.

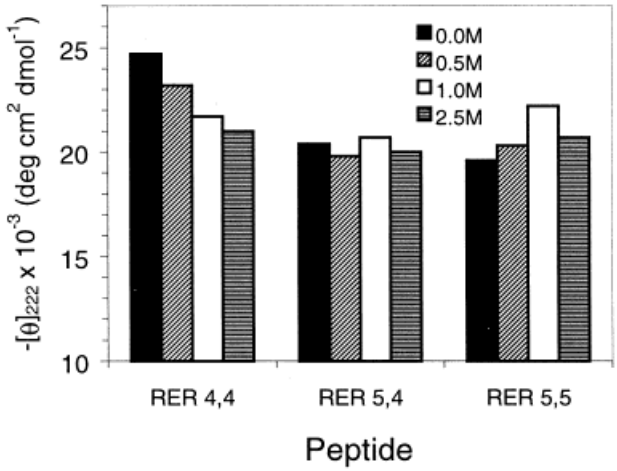

Fig. 2. Comparison of the effect of increasing NaCl concentration on the salt bridge interactions in model peptides: a complex salt bridge (RER4-4), a simple salt bridge (RER5-4), and the reference peptide (RER5-5).

water and because its methyl side chain minimizes interactions with adjacent charged Arg and Glu side chains (see, e.g., Marqusee and Baldwin[39]). Placing ornithine residues at each end of the peptides enhances solubility and limits interactions of the basic residues with the core of the helix.[23] The Orn groups are positioned at least five residues away from the core Arg side chains to minimize the possibility of interaction. At low salt, it is likely that these groups also interact with the core of the helix. A tyrosine residue introduced at the C-terminus facilitates concentration determination. All peptides are acetylated at the N-terminus and capped with an amide at the C-terminus to minimize fraying and reduce the helix dipole effect.[4,40]

CD Analysis

All peptides of this series show strong α-helical CD signals, with characteristic minima at 222 and 208 nm. An isodichroic point is seen at 203 nm for all peptides in the series, characteristic of a two-state transition for each residue (Fig. 1; Table II).The CD signal of the peptides is independent of concentration from 10–400 mM (data not shown), consistent with the helical structure being intramolecular and not due to association. A peptide of similar design lacking the core Arg and Glu side chains was previously shown to be monomeric in an analytical ultracentrifugation experiment.[23]

The peptides containing putative salt bridges show increased helicity relative to the control (RER5-5), the peptides with multiple salt bridges being more stable than those with single salt bridges (Table II). The differences in helix content have been converted into free energies as described in Materials and Methods, using a helix–coil transition model to deconvolute the CD data. The NMR results described below suggest that the N→C acid-base orientation is slightly more stabilizing than the opposite orientation. The differences in helicity between peptides with single salt bridges spaced at intervals of $i\pm3$ or 4 do not reveal this trend, possibly because the differences are close to the error limits. Peptides containing complex salt bridges consistently show higher helix content than those with single bridges (Table II). The increase in helicity is additive as judged from the helix contents except in the case of RER4-4, where the stabilizing interaction between the salt bridge triads exceeds the additive contributions to stability from two simple ion pairs. Thus, the helix content of RER4-4 is 15% more than in RER5-5. Adding the differences between RER4-5 and RER5-4 and the reference peptide, the single bridge contributions to helix content are 6.5 and 3%, respectively. Thus, RER4-4 has higher helix content than predicted by the sum of the individual bridges in the latter peptides. This is apparent in the resulting free energy as expected.

Of the four complex salt bridges studied, the symmetrical RER4-4 arrangement seems to be the most stabilizing followed by RER3-3. Since the natural alpha helix period is closest to seven residues, we anticipated that the two peptides with mixed spacing, RER3-4 and 4-3, would be more helical than either RER3-3 or RER4-4. This is not the case. We attribute the difference between RER4-4 and RER3-3 and the other combinations to more favorable side chain orientational effects in the former.

Salt Concentration Dependence

Neutral salts were added to the peptide solutions to assess the dependence of salt bridge interactions on ionic strength. In the reference peptide, clear effects of added salt can be seen in the CD spectra (Fig. 2): helicity increases with added neutral salt up to a concentration of 1M, as has been found previously.[4,42] The reduction in helix content from 1M to 2.5 M salt is attributed to the effect of the salt on water, rather than to charge screening. Weaker effects of added salt are observed for single salt bridge peptides (Fig. 3). The effects reduce the trend seen in the reference peptide, indicating loss of stability as salt concentration increases up to 1M. In the case of the complex bridge in RER4-4, a destabilizing trend with salt concentration is seen. Multiple salt bridges are more sensitive to screening than simple bridges, since there are more interacting charged groups in proximity. A set of

TABLE II. Observed Helicity and Energetics of Salt Bridges

Peptide	$-[\theta]_{222} \times 10^{-3}$ (deg cm^2 dmol^{-1})[a]	f_H[b]	$-\Delta\Delta G$ (kcal mol^{-1})[c]	$-\Delta\Delta G$ for complex bridges (kcal mol^{-1})
RER4-4	24.7	0.72	2.36	1.5
RER3-3	24.0	0.70	2.19	1.0
RER3-4	23.3	0.68	1.35	0.3
RER4-3	23.0	0.67	1.13	0.1
RER3-5	22.2	0.65	0.803	
RER4-5	21.7	0.635	0.623	
RER5-3	21.3	0.62	0.409	
RER5-4	20.6	0.60	0.223	
RER5-5	19.6	0.57	0.00	

[a]In 10 mM phosphate buffer pH 7 at 4°C. The peptide concentrations are 50 μM as determined by tyrosine absorbance at 275 nm.
[b]The relationship between fraction helicity and molar ellipticity is $f_H = -[\theta]_{222}/34{,}000$, where −34,000 is the estimated molar residue CD signal at 222 nm for an α-helix of 24 residues.
[c] The free energies of side chain interactions were computed using an algorithm based on the Zimm–Bragg helix–coil transition model, with the nucleation parameter $\sigma = 0.004$ and values of the helix propensities for amino acids Ala, Glu, Arg, Phe, and Orn.

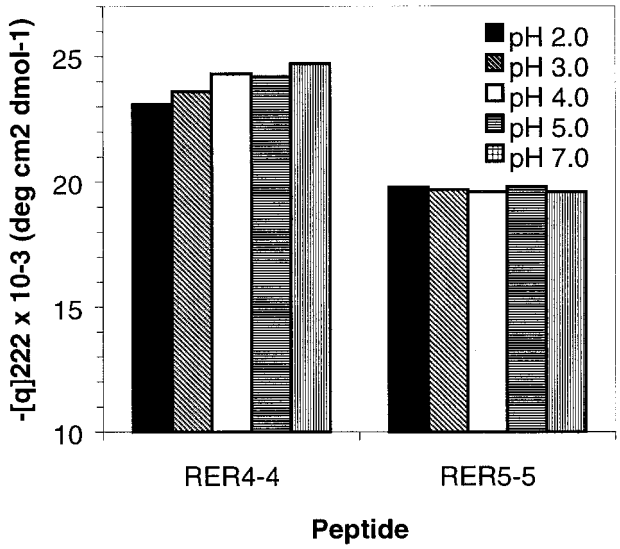

Fig. 3. Comparison of helicity of RER4-4 and the reference peptide RER5-5 showing the effect of titrating Glu on the strength of the interaction between Glu and Arg.

eight salt bridge pairs in a single peptide, for example, was found to be destabilized almost completely by added salt.[3] Nevertheless, the enhanced helicity in RER4-4, for example, persists at 0.5M salt, indicating that the terminal Orn side chains are not playing a role.

pH Dependence

By protonating the central Glu residue, we can discriminate the contributions to the Arg–Glu–Arg interaction from hydrogen bonding and from ionic attraction. We compared the helicity of RER4-4 and RER5-5 at several pH values near the pKa of Glu. Figure 3 shows the results. The molar ellipticity of RER5-5 increases only slightly from pH 7 to pH 2. This is consistent with a small increase in the helix propensity of uncharged glutamic acid relative to that of glutamate.[42] The decrease in mean molar ellipticity of RER4-4 can be attributed to the loss of the stabilizing charge interaction between the positively charged arginines and the central glutamate. As in the case of binary salt bridges,[3,4] this decrease in helicity indicates that a loss in charge does not eliminate the stabilizing interactions present in the triad. The difference in helix content between RER4-4 with a protonated Glu and RER5-5 shows that hydrogen bonding, in fact, accounts for much of the stability of the salt bridge interaction.

NMR Analysis

It is important to establish that the salt bridges postulated to form in these peptides are actually present. This requires a higher resolution structural analysis. We recorded the ^{1}H TOCSY(80 ms) and NOESY(400 ms) spectra of three peptides of the series: RER5-5, RER5-4, and RER4-4. Proton chemical shifts for the peptides were assigned following standard procedures, combining the TOCSY and NOESY data.[33] Figure 4 shows the aliphatic-amide region of the NOESY spectrum of RER4-4, with the partial assignments of the RER4-4 spectrum. Proton chemical shifts for all the non-alanine residues and majority of 16 alanines (10 out of 16) are assigned. Because of the degeneracy of the α-protons and the methyl groups of the alanine residues, some of these residues cannot be assigned; assignment of all residues other than alanine is unambiguous. Figure 5 shows all the assigned NH_i-NH_{i+1} cross-peak connectivities, which together with low values of $^3J_{NH}$ coupling constants, which are from relatively small line width and weak cross peaks between amide and α- protons in the TOCSY spectra, is characteristic of α-helical conformation.

The hydrogen exchange rates of the Arg guanidinium group also reflect the strength of their interactions with a neighboring Glu side chain.[43] Rates of H-exchange can be determined by ^{1}H NMR magnetization transfer[44]; the water signal was pre-saturated for increasing lengths of time (t_{sat} ~50 msec to 3 sec) during which the initial NH resonance intensity (I_o) decreased as in Eq.(1)[45] due to

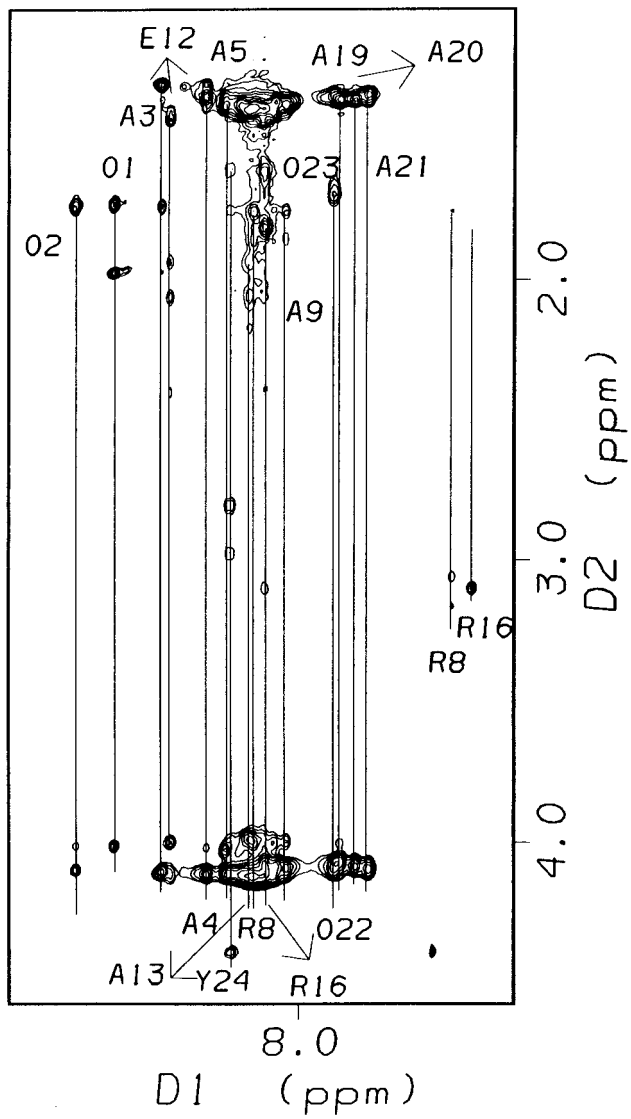

Fig. 4. Aliphatic-amide region of the NOESY spectrum of RER4-4 at approximately 5.0 mM at 5°C with a 400-ms mixing time, indicating assigned amide protons and their cross peaks with other aliphatic protons.

exchange with the saturated H_2O protons, ultimately attaining a steady state value at times long relative to the spin-lattice relaxation time (T_1) and exchange lifetime:

$$I(t_{sat}) = I(t_o)[(k_{ex}/k_s)\exp(-k_s t) + (1/K_s T_1)] \quad (1a)$$

$$k_s = k_{ex} + 1/T_1 \quad (1b)$$

The apparent k_{ex} values are: 0.04 ($\pm 0.4\ s^{-1}$), 0.21 ($\pm 0.3\ s^{-1}$) for R8 and R16 in peptide RER4-4, vs. 0.56 ($\pm 0.3\ s^{-1}$) and 1.25 ($\pm 0.4\ s^{-1}$), the averages of the corresponding two Args in RER5-4 and RER5-5, respectively (see Fig. 6). The rates cited are apparent values, because corrections for the T_1 contributions were not applied, and the data for RER5-4 and RER5-5 were fit to single exponentials rather than to a double exponentials. The trend is clear, however: the exchange rates of the two Arg side chains in RER4-4, R8 and R16, are slowest, those in RER5-5 are fastest, while those of RER5-4 are intermediate.

The dependence of the chemical shift of an NH proton on temperature provides a further indication of its state of internal H-bonding.[46–48] We measured shift values of −4.6 ppb/K and −5.7 ppb/K for R16 and R8 in RER4-4, −5.6 ppb/K as an average for R7 and R16 in RER5-4, and −6.2 ppb/K as an average for R7 and R17 in RER5-5. These data are consistent with the idea that side chain–side chain interactions are stronger in RER4-4 than in RER5-4, and stronger in both of these than in RER5-5. The NMR experiments indicate that the interaction in the E-R orientation is stronger than in the R-E orientation in agreement with earlier results[4,39] although, as noted, we could not detect this in the CD data on single bridge peptides. Several factors come into play in this effect: one is that long side chains in a helix preferably orient towards the N terminus.[49] This is likely to favor configurations in which the longer side chain is C terminal to the shorter one. In addition, the E-R orientation can make use of the preferred side chain rotamers for both E and R, while the interaction in the R-E orientation requires non-preferred side chain rotamers.[24] The NMR data confirm that side chain–side chain interactions are strong in RER4-4 but not in RER5-5, and weaker in RER5-4.

DISCUSSION

Experimental and theoretical analysis of the role of ion pairs or salt bridges on the stability of proteins has so far failed to provide a coherent picture of their quantitative effects (see Nakamura[21]). Surface or internal salt bridges appear to contribute little to protein stability.[6,7,10,50] A notable exception is the case of an internal salt bridge in T4 lysozyme, which was reported to have a substantial stabilizing effect, in excess of 2 kcal/mol.[5] Binary interstrand salt bridges have been shown to stabilize coiled–coil helical structure.[51,52] Proteins from hyperthermophilic organisms contain more surface salt bridges than those from other organisms, and extensive networks of salt bridges have now been reported in several such proteins.[8] Since ionic interactions can extend over considerable distances on the surface of proteins depending on the ionic strength,[53,54] one would like to understand the roles of neighboring ions as well as those of the side chains directly participating in bridges.

The availability of helical peptides rich in Ala side chains makes it possible to investigate side chain–side chain interactions in a simplified context. Our previous analysis of the role of complex salt bridges in the GCN4 coiled-coil showed a clear stabilizing effect, but the presence of charged side chains at the proximal *e* and *g* positions in the molecule influence the interactions at the *b* and *f* sites that were used.[12] In the present peptides, the Ala side chain stabilizes α-helix and minimizes many if not all inter-side chain effects.[39] Ala arguably provides an ideal test bed for introducing sets of potentially interacting residues into a helix, and Baldwin's group has shown that Ala rich peptides have played a major role in elucidating the mechanism of helix formation in water.[14,37] Since

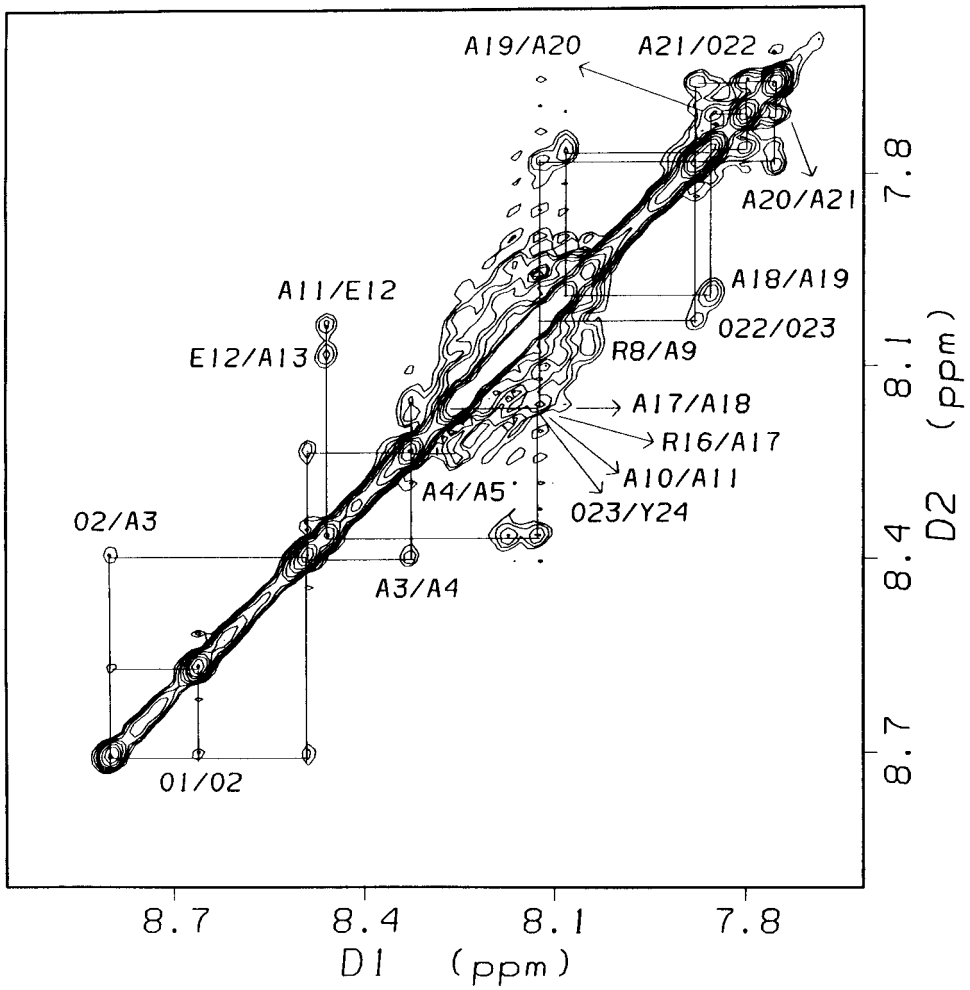

Fig. 5. Amide-amide region of the NOESY spectrum of RER4-4, indicating cross peaks between backbone amide protons. Sequential cross peaks are indicated by the numbers of the residues.

peptides containing only Ala are insoluble in water, it has been necessary to introduce polar or charged side chains to ensure solubility of the chains in water. In the studies of Scholtz and coworkers using a $(AAQAA)_n$ host peptide, for example, the location and hydrogen bonding capability of the glutamine side chain can influence neighboring charged residues.[18,19,42] The effect of position and orientation of Arg and Glu in isolated helical peptides has been studied previously by Huyghues-Despointes et al.[18] who introduced sets of these side chains into a background of Ala, a design that is similar to the present one. They reported strong and selective salt bridge interactions between the side chains.

It has been recognized for some time that tri-block copolymers of the form Lys_n–Ala_m–Lys_n solubilize segments of alanine residues[55]; the design we use here includes only two charged side chains at each end.[23] (Reduction to a single terminal charged side chain[56] produces stable β sheet formation.) There are no gratuitous polar side chains in the interior of the helices of this study. The helicity of the series of peptides increases depending on the number of side chains that can interact (Table I). To extract free energy values, our analysis makes use of a Zimm–Bragg type of helix–coil transition model[35,36] with helix propensities and nucleation constants that reproduce the observed helix content in RER5-5. The values used to describe the helical structure in RER5-5 are obtained from the literature.[23,36–38] We introduce a second statistical weighting factor γ whenever three or four helical residues can be linked by a salt bridge. Values of γ are varied to minimize the standard deviation between calculated and measured helix contents. In this way, we fit apparent γ constants to the CD spectral data for each of the 8 peptides that allow formation of salt bridges. The value for the RER4-4 bridge exceeds those for any of the simple pairs or other sets of complex bridges studied (Table II). The overall stabilization attributable to this triadic bridge is ~2.4 kcal/mol, in the same range as the value we reported previously for a complex RER bridge in a coiled-coil helical model, 1.7 kcal/mol.[12] However, the latter case corresponds to a (3,4) spacing of the charged side chains conforming to the heptad repeat in coiled coils. The (4,4) and (3,3) spacings in the peptides of the present study show the strongest deviation from simple additivity

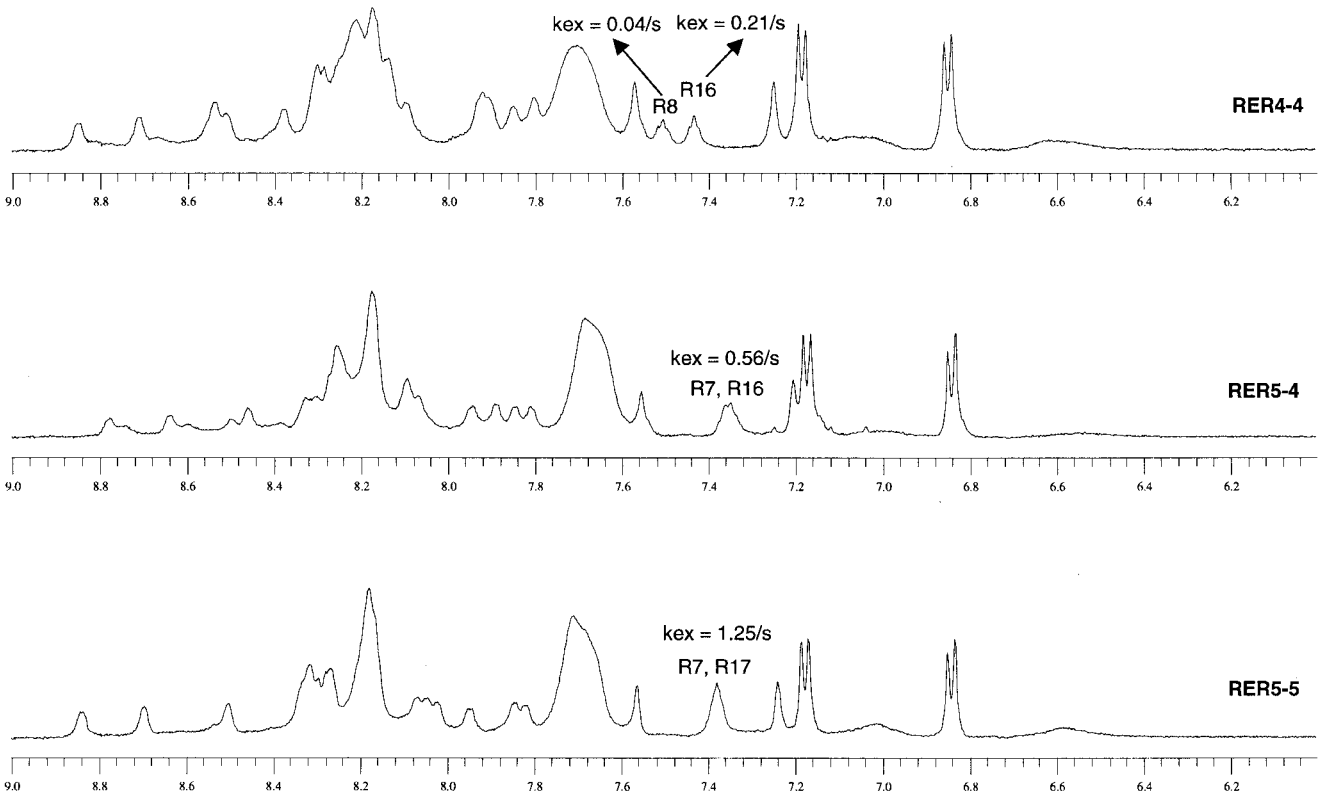

Fig. 6. Amide and aromatic regions of 1D proton spectra of RER4-4, RER5-4, and RER5-5 at 5°C. The assignments of Arg side chain guanidinium groups and the corresponding k_{ex} are also shown in the spectra.

of the contributions from pair-wise bridges. The (3,4) and (4,3) spacings in the present peptides are weaker. Since Arg and Glu are both long side chains, one possibility is that in isolated helices the (4,4) spacing permits optimal interaction among the side chains. Huyghues-Despointes et al.[18] reported the order of stability at pH 7 to be: i+4 ER > i+4 RE > i+3 ER > i+3 RE. Their model consisted of 3 adjacent sets of salt bridges and showed larger effects than we find in our single salt bridge peptides, where the differences in our CD spectra are small (Table I). The geometry of Arg–Glu interactions in native proteins has been analyzed by Thornton's group, who identify a variety of patterns of interaction, including both side-by-side and end-on orientations of the head groups.[57] This diversity in geometry helps to explain the differences we see among spacings in the side chains of these peptides.

What is the basis for the non-additive enhancement of stability in RER3-3 and RER4-4? We believe one contribution is an entropic effect.[58] If strain or conformational restrictions are absent, forming a complex bridge entails ordering three side chains rather than four in two independent bridges. This effect would be predicted to strengthen as the cluster size and complexity increase, and in larger numbers could play a significant role in protein stabilization. The glutamate dehydrogenase from *Pyroccus furiosus,*[8] for example, contains an 18-residue ion pair network that includes 14 sets of RER or ERE side chain triads. With no additional stabilizing effect from higher connections among triads, a contribution in the range of 6–12 kcal/mol could exist (depending on the combinations that are spaced correctly), sufficient to ensure thermostability of the enzyme. How the spacing and higher connectivity in more complex salt bridges affect protein stability remains to be determined.

Substitution experiments on salt bridges raise a number of further questions concerning the nature of the reference state of the protein or peptide, the role of the helix dipole and terminal charges, as well as the contribution of van der Waals interactions in stabilizing the interaction. Use of peptides of identical composition minimizes charge and substitution differences that change the reference state.[7,24,54] The calculations by Luo et al.[54] rely on an oligo (Ala) helical peptide that is very similar to that of this study, apart from the terminal Orn side chains. Their analysis predicts no specific conformational effects in forming simple salt-bridges, although, as pointed out, the Arg–Glu interaction appears to show strong orientational effects.[18] These are not in the same direction as those in His-Asp models.[24] We selected Arg as a participant because it is found most frequently in salt bridges of proteins from thermophiles, presumably because the larger charged Arg surface facilitates ion pairing relative to Lys, while its greater basicity ensures that it retains a positive charge at elevated temperatures.

An important factor in the electrostatic interactions of model peptides is the potential for longer range ionic interactions in a given chain. A neutral α-helix possesses a dipole moment directed along the axis, with the N termi-

nus being positive and the C terminus negative.[59] Arrangements of charged groups that diminish the helix dipole stabilize helical structure and vice versa.[40] This may be one explanation for the general trend reported by Baldwin's group that the AB orientation of acidic and basic groups tends to be more helix stabilizing than the reverse BA.[24,39] In our models, the two positive charges at each terminus may also affect the helicity. In a low salt environment, these can reasonably be expected to interact with the internal RER groups; however, at 0.5M salt (see Fig. 2) where the Debye screening length is severely reduced, this is unlikely. Hence, we believe the effects we are studying reflect local interactions among the RER side chains rather than differential effects arising from the role of Orn side chains at the ends. Despite the fact that the Glu side chain is central in each peptide sequence, helix dipole interactions or interactions from charges at the ends may still play some role in our models. The analysis of the apparent dipolar field in an alpha helix by Scholtz et al.[42] indicates that the field is in fact asymmetric with respect to the mid-point of the sequence. Adding the Orn groups might enhance this effect. The helix distribution tends to be asymmetric in most peptides, tending to favor the N terminus rather than the C terminus, possibly reflecting the greater strength of N-capping interactions.[60]

Finally, it should be noted that the role of van der Waals interactions in stabilizing side chain–side chain interactions in Ala-rich peptides is probably appreciable. A stabilizing interaction between the methyl groups of Ala residues in a helix and methylene groups of the Lys side chain was predicted many years ago and detected recently.[61] van der Waals interactions of methylenes of Arg with the helix barrel of Ala, in principle, stabilize simple or complex salt bridges. A van der Waals contribution to differences in helicity between bridges spaced at i,i+3 and i,i+4 as well as to differences in orientation between Glu-Arg and Arg-Glu may contribute to the enhanced stability seen in RER4-4 relative to the other peptides of this study.

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