

## Stabilizing Effect of a Multiple Salt Bridge in a Prenucleated Peptide

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Received May 7, 1998

**Abstract:** The free energy contribution of salt bridges to protein stability remains a controversial issue. The role of single salt bridges has been investigated in protein and peptide studies; there is no reported value of a multiple salt bridge employing peptide models. We have designed a de novo peptide model to evaluate the strength of a multiple salt bridge. Measurement of the rate of hydrogen exchange (HX) of the backbone NH groups in  $\alpha$ -helical peptides allows determination of the absolute free energy of individual interactions that participate in helix stabilization. We apply this procedure here to evaluate the stabilizing contribution of a multiple salt bridge involving Glu3, Asp4, and Arg7 in an 11-mer peptide. The peptide is pre-nucleated by a lactam bridge connecting two side chains spaced four residues apart. Measurement of the HX rates of each NH group in this peptide as a function of pH, together with the pH independent rate of a Gln3 and Asn4 analogue, reveals a substantial favorable contribution to the  $\alpha$ -helix from the salt bridge:  $\Delta G^\circ = -1.2 \pm 0.2$  kcal/mol. This is the first evaluation of the strength of an engineered complex salt bridge in a peptide and yields a high value, comparable to results of mutation studies on proteins.

Hydrophobicity, van der Waals packing, hydrogen bonds, and electrostatic contributions of salt bridges and helix–dipole interactions have all been implicated in stabilizing the native state of proteins against unfolding. To date there is no consensus on the quantitative contribution of these interactions. Peptide models can simplify the evaluation of the free energies of individual interactions responsible for protein stability. These models suggest a stabilizing strength of about 0.5 kcal/mol for a single salt bridge in a helix, at 0 °C and low ionic strength.<sup>1,2</sup> Mutational studies on T4 lysozyme protein report different values. A single salt bridge within the hydrophobic core of the protein was found to be stabilizing by 3–5 kcal/mol<sup>3</sup> in contrast to salt bridges engineered on the surface of a solvent-exposed helix in the same protein which are only marginally stabilizing.<sup>4</sup> Here we are concerned with the strength of multiple salt bridges rather than simple ion pairs. Two mutational studies on proteins have reported on the strength of a multiple salt bridge. A salt bridge triad between an arginine and two aspartates on the surface of barnase stabilizes the protein by 1.25 and 0.98 kcal/mol, respectively.<sup>5</sup> If any of the interactions in the triad is removed, the overall stabilization diminishes by 0.77 kcal/mol, a strong coupling effect. A salt bridge–hydrogen bond network in lambda repressor is reported to be stabilizing by 0.8 and 1.5 kcal/mol, respectively. This network is partially buried, however.<sup>6</sup>

We address the question of the strength of a multiple salt bridge by constructing the following peptides:<sup>7</sup>

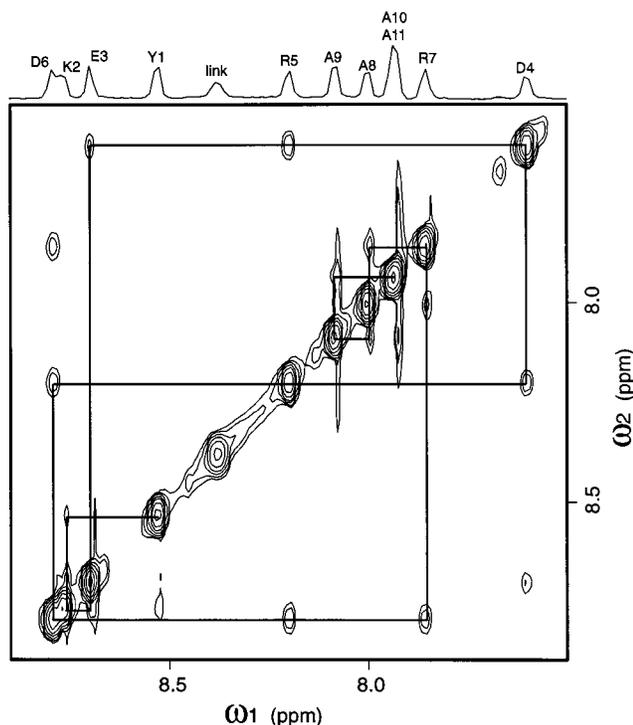
A: ac-YKEDRDRAAAAA-NH<sub>2</sub>B: ac-YKQNRDRAAAAA-NH<sub>2</sub>

Each peptide is pre-nucleated by a covalent lactam bridge between Lys2 and Asp6, promoting  $\alpha$ -helical structure. The helicity is further enhanced by blocking both ends of the chain; the N terminus is acetylated, while the C terminus is amidated. A tyrosine residue is introduced to facilitate concentration determination. The potential complex salt bridge is introduced by Arg7 and Glu3, Asp4 in peptide A. Peptide B cannot form this salt bridge since the acidic residues are replaced by Gln and Asn.

Salt bridges include a hydrogen bond as well as an ionic component. The strength of the ionic component can be measured by pH titration. Lowering the pH will screen the negative charges, thereby nullifying the ionic component of the salt bridge. The difference in stabilization at neutral and low pH can therefore be taken as a measure of salt bridge strength. Free energy values at various pHs can be assigned roughly by deconvoluting the CD spectrum in peptides.<sup>8</sup> A more accurate method is to use NMR and measure the hydrogen exchange rates of the peptide group in the  $\alpha$ -helix.<sup>9</sup> This allows us to evaluate the free energy value for each specific site. The strategy is to measure the exchange rate of each individual peptide NH in a helical peptide, refer this rate to a control in which there is no helical structure, determine the protection

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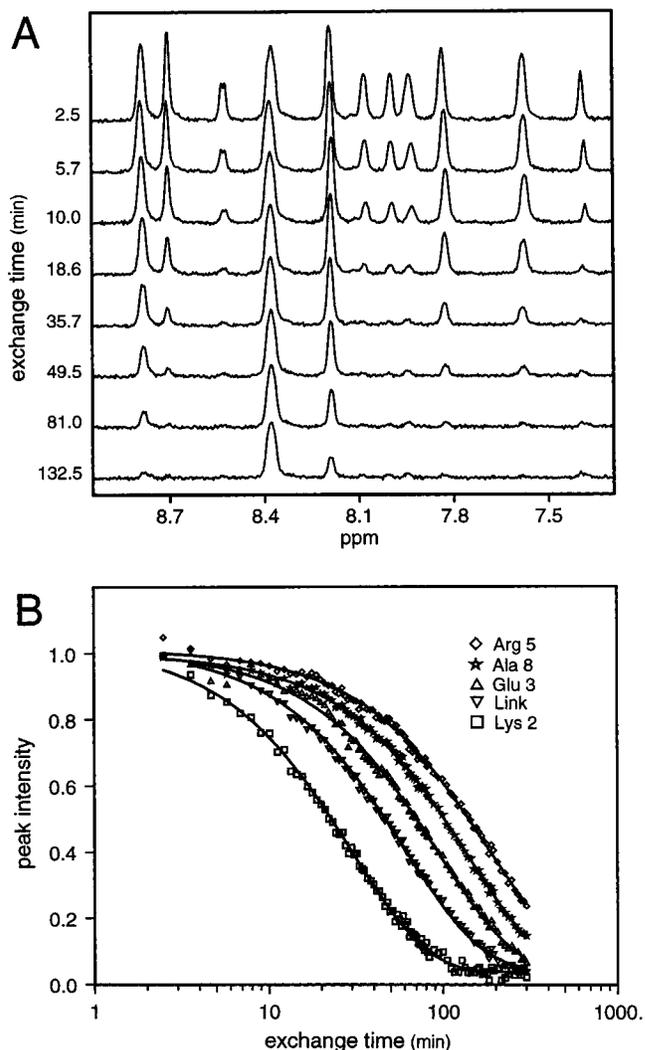
**Figure 1.** NH–NH region of the NOE spectrum of Peptide A at 1 mM concentration and 5 °C, showing successive  $i, i + 1$  NH–NH interactions indicative of helical structure.

factor  $P$ , the ratio of these rates, and thereby determine  $\Delta G_{\text{HX}}(i) = RT \ln(P_i - 1)$ , the effective free energy corresponding to the ease or difficulty of the opening reaction at each site in the chain. We have shown previously that these values provide free energy values that reflect all of the stabilizing interactions affecting the backbone NH group at site  $i$ .<sup>7</sup>

## Results and Discussion

CD spectra were measured at pH = 7 for each of the peptides at 4 °C in low salt (not shown). The spectra show an  $\alpha$ -helix signature with  $[\theta_{222}]$  values of  $-16\,500$  and  $-15\,000$  for A and B, respectively. The  $\alpha$ -helix signature was also observed in the NMR spectra. Assignment of the  $^1\text{H}$  NMR spectrum of peptide A followed the side-chain–main-chain procedure based on 2D TOCSY and NOESY experiments. The assignment network is shown in Figure 1. Sequential  $\text{NH}_i, \text{NH}_{i+1}$  NOE connectivities define a helix extending from residue 1 to residue 10 (overlapped in shift with residue 11) at the C terminus. Only small changes in chemical shift are detected in the amide region as a function of pH. The extra-helical peptide NH group in the lactam bridge is also monitored, and HD exchange of this group can be determined along with that of the main-chain NH groups. The  $^1\text{H}$  NMR spectrum of peptide B has very similar characteristics (data not shown).

The hydrogen exchange measurements are represented in Figure 2. Panel A illustrates the NH region of the spectrum of peptide A as a function of time after shifting a sample in  $\text{H}_2\text{O}$  to  $\text{D}_2\text{O}$ ; a profile of the exchange of several residue NHs is shown in Figure 2B, plotted on a logarithmic time scale. The data in Figure 2 show that the individual hydrogen-exchange rates in these peptides can be determined precisely.<sup>9</sup> The measured exchange rates,  $k_{\text{obs}}$ , can be compared to the intrinsic chemical exchange rate,  $k_{\text{chem}}$ , for an unstructured peptide of



**Figure 2.** Representative hydrogen exchange measurements of peptide A at 1 mM concentration and 5 °C. (A) The amide region of NMR spectra as a function of time after dissolution into  $\text{D}_2\text{O}$ . (B) Integrated peak intensity as a function of exchange time for several residues plotted on a logarithmic time scale. The solid lines are single-exponential fits to the data.

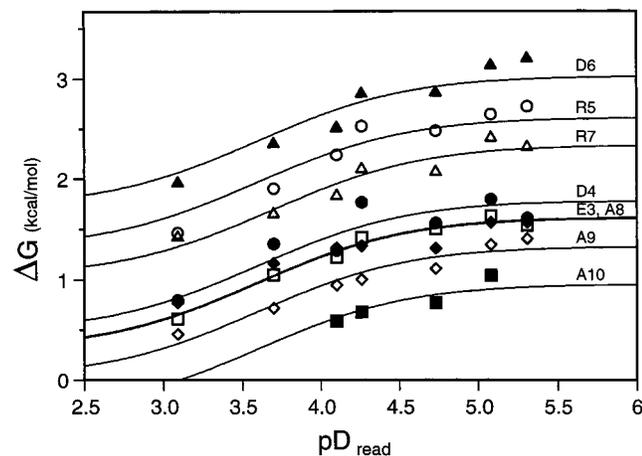
the same sequence<sup>10</sup> to assess individual protection factors ( $P_i = k_{\text{chem}}/k_{\text{obs}}$ ) and the corresponding free energies of opening,  $\Delta G_{\text{HX}}(i) = RT \ln(P_i - 1)$ . The results are shown in Table 1 as a function of pD. Figure 3 shows the effect of pD on exchange rates and corresponding opening free energies of the individual NH groups in peptide A. The protection factors vary with position in the sequence<sup>7</sup> and also depend on pD. The  $P_i$  values appear to shift collectively with an apparent  $\text{p}K_{\text{a}} = 4.1$  ( $\text{D}_2\text{O}$ ) and a  $\delta\Delta G_{\text{HX}}$  of 1.2 kcal/mol. The only titratable groups in this pH range are the side chains of Asp3 and Glu4. This strikingly large stabilizing value upon changing the pH can be explained in terms of a model in which the Arg7 side chain interacts with both the Glu side chain and the Asp side chain, forming a multiple salt bridge. The pH dependence is not seen in peptide B containing the Gln3, Asn4 variant. This provides direct evidence for an Arg to Glu/Asp salt bridge in peptide A. As expected, peptide B shows no change in stability as the pH is lowered (data not shown).

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**Table 1.** Protection Factors and Free Energies of Peptide A

residue	pD <sup>a</sup> = 5.31		pD = 3.09	
	P <sup>b</sup>	ΔG (kcal/mol)	P <sup>b</sup>	ΔG (kcal/mol)
Y1	1.51	-0.37	1.02	-2.2
K2			0.60	
E3	17.1	1.54	4.03	0.61
D4	19.1	1.61	5.23	0.80
R5	139	2.72	15.1	1.46
D6	330	3.20	35.4	1.96
R7	67.1	2.32	14.0	1.42
A8	18.0	1.57	5.04	0.77
A9	13.7	1.14	3.30	0.46
lactam	7.34	1.02	134	2.70

<sup>a</sup> pD = uncorrected pH meter reading in D<sub>2</sub>O. <sup>b</sup> P = Protection factor ( $k_{\text{chem}}/k_{\text{obs}}$ ).



**Figure 3.** Free energy of hydrogen exchange as a function of pD for the hydrogen bonded peptide NHs. The curves are the result of a global fit to a simple pH titration expression which uses a single  $pK_a$  of 4.1 and a  $\Delta\Delta G_{\text{HX}}$  of 1.2 kcal/mol for all residues yet allows each residue to have its own  $\Delta G_{\text{HX}}$  (vertical position) in the high pH form.

A salt bridge can be described in terms of two components, an ionic interaction and a hydrogen bond. Upon the pH being lowered, the ionic interaction is lost, but the hydrogen bond remains intact. The titration data thus imply that the ionic component alone accounts for 1.2 kcal/mol of helix stabilization. The H-bond contribution of a single solvent-exposed ion pair has been estimated to be 0.3 kcal/mol.<sup>11</sup> The complete multiple salt bridge is thus likely to be helix-stabilizing more than 1.2 kcal/mol. In this total, the helix-dipole interaction has to be taken into account. A negatively charged residue near the N terminus stabilizes the  $\alpha$ -helix. This effect is lost at acidic pH. The 1.2 kcal/mol stabilization should therefore be attributed both to the salt bridge and the helix-dipole interaction. The helix-dipole interaction has been quantitatively investigated by Huyghues-Despointes et al.<sup>12</sup> Converting their data from  $\Delta pK_a$  values gives a helix-dipole stabilization of 0.25 kcal/mol for the two side chains together. Taking into account both the hydrogen bond component of the salt bridge and the helix-dipole interaction, the strength of the multiple salt bridge is  $1.2 \pm 0.2$  kcal/mol.

The 1.2 kcal/mol stabilization we find is well above the free energies estimated for single solvent-exposed salt bridges in either peptide<sup>1,2</sup> or protein studies.<sup>4</sup> It is comparable to the values derived for a multiple salt bridge in barnase<sup>6</sup> and suggests

that summation of multiple salt bridge free energies can provide a major contribution to native protein stability, as has been proposed from studies of proteins from hyperthermophiles.<sup>13</sup>

## Experimental Section

**Peptide Synthesis and Purification.** Peptides were synthesized manually by solid-phase peptide synthesis on MBHA (*p*-methylbenzylamine-polystyrene) resin using the *t*-Boc/OBzl protection strategy. The protecting groups for the side chains of Lys2 and Asp6 which form the *i, i + 4* lactam bridge were Fmoc (Lys) and For (Asp). The *t*-Boc was removed by 30% TFA/5% anisole in DCM for 2 min, the process was repeated for 30 min. Neutralization was accomplished using 10% diisopropylethylamine (DIEA) in DMF. For each coupling step a 5-fold excess of amino acid was used preactivated by BOP/HOBt in DMF containing 0.2 M DIEA. The coupling time is 2 h. The Lys and Asp protecting groups were removed by using piperidine/toluene/DMF (30/35/35%). Lactam-bridge formation was carried out using BOP/HOBt/DIEA (10/10/5 equivalent) in DMF overnight. The N-terminal amino group was acetylated using acetic anhydride/DIEA in DMF. Peptide was cleaved with HF/*p*-cresol/anisole at 0 °C for 1 h. The crude product was washed with cold ether and dissolved in 10% acetic acid. The crude peptide was purified by RP-HPLC on an Econosil C18-10  $\mu\text{m}$  column (250  $\times$  22.5 mm) monitored at 275 nm with a gradient of acetonitrile in 0.1% trifluoroacetic acid. The purity was checked on a Waters analytical column (Delta Pak C18-100 Å). The correct masses of the peptides were confirmed by electrospray ionization mass spectroscopy, using a Vestec model 201 mass spectrometer.

**Circular Dichroism Spectroscopy.** CD measurements were conducted on an Aviv DS 60 spectropolarimeter equipped with an HP 89100A temperature controller. The instrument was calibrated with (+)-10-camphorsulfonic acid.<sup>14</sup> All measurements were performed in 10 mM KF, 1 mM phosphate buffer pH 7 at 4 °C. The concentration was determined by the tyrosine UV absorption with maximum specific absorbance at 275.5 nm of 1450 at 25 °C in 6.0 M GnHCl. The CD measurements were carried out at peptide concentrations of 30  $\mu\text{M}$ , averaging over three scans with a step size of 0.5 nm.

**NMR Spectroscopy.** The <sup>1</sup>H NMR spectra were assigned using combined TOCSY and NOESY 2D NMR experiments. Hydrogen-exchange experiments were acquired by 1D <sup>1</sup>H NMR using a Bruker AM 500 MHz spectrometer.<sup>7</sup> The peptides were initially lyophilized from buffered H<sub>2</sub>O solutions and dissolved directly into D<sub>2</sub>O. Samples were then pipetted into NMR tubes and a series of 1D spectra recorded to follow the time-dependent loss of the NH resonances.

**Data Analysis.** Hydrogen-exchange rate constants were divided by the factors representing effects of neighboring sequence on exchange to obtain protection factors,  $P_i$ , at each NH position,  $i$ , in the chain.<sup>10</sup> These factors are inversely related to the local or global unfolding probability that the NH is exposed to solvent rather than H-bonded.<sup>9</sup> The free energy of stabilization of the helix itself is measured by the HX rate at the site that has the largest  $P_i$  factor overall (Asp6). The helix in peptide A is stabilized by 3.2 kcal/mol relative to an unstructured peptide of the same sequence. Changes in the  $P_i$  values with pD were plotted in Figure 3, using the free energy scale  $\Delta G_{\text{HX}}(i) = RT \ln(P_i - 1)$ . The lines in the figure illustrate the expected dependence of the free energies on pH according to the Henderson-Hasselbach equation modeled for a single  $pK_a$  value of 4.1. The shift in  $P_i$  values at all sites is then well-accounted for by a single ionization process with this  $pK_a$  value, and a free energy differential of about 1.2 kcal/mol.

**Acknowledgment.** This research was supported by NIH grants DK11295 (L.M.) and GM 40476 (N.R.K.) and a grant from the Human Frontiers of Science Program.

JA981592C

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