

Helical peptides with three pairs of Asp-Arg and Glu-Arg residues in different orientations and spacings



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Abstract

The helix-stabilizing effects of repeating pairs of Asp-Arg and Glu-Arg residues have been characterized using a peptide system of the same design used earlier to study Glu-Lys (Marqusee, S. & Baldwin, R.L., 1987, *Proc. Natl. Acad. Sci. USA* 84, 8898–8902) and Asp-Lys ion pairs (Marqusee, S. & Baldwin, R.L., 1990, In *Protein Folding* [Gierasch, L.M. & King, J., Eds.], pp. 85–94, AAAS, Washington, D.C.). The consequences of breaking ion pair and charge–helix dipole interactions by titration to pH 2 have been compared with the results of screening these interactions with NaCl at pH 7.0 and pH 2.5. The four peptides in each set contain three pairs of acidic (A) and basic (B) residues spaced either $i, i + 4$ or $i, i + 3$ apart. In one peptide of each kind the pairwise order of residues is AB, with the charges oriented favorably to the helix macrodipole, and in the other peptide the order is BA.

The results are as follows: (1) Remarkably, both Asp-Arg and Glu-Arg peptides show the same pattern of helix stabilization at pH 7.0 found earlier for Glu-Lys and Asp-Lys peptides: $i + 4 AB > i + 4 BA \approx i + 3 AB > i + 3 BA$. (2) The ion pairs and charge–helix dipole interactions cannot be cleanly separated, but the results suggest that both interactions make important contributions to helix stability. Because the four peptides of each set have nearly identical compositions, the large differences in helix content within each set cannot be caused by differences in length and sequence. (3) Significant differences in helix content remain at 5 M NaCl, where ion pair interactions are completely screened. The residual interactions may either be nonscreenable H-bond interactions or remaining charge–helix dipole interactions.

Keywords: α -helices; charge–helix dipole interaction; ion pairs

The four Glu-Lys and the two Asp-Lys peptides studied by Marqusee and Baldwin (1987, 1990) have nearly the same amino acid compositions (the 16- and 17-residue peptides differ by one alanine residue) and yet show very different helix contents, both at pH 7.0 and at pH 2.5. Consequently, ion pair interactions and charge–helix dipole interactions make important contributions to helical stability. Since the initial report by Marqusee and Baldwin (1987), several other groups have demonstrated the importance of electrostatic interactions in the stability of α -helical peptides. Merutka and Stellwagen (1991) have investigated the effects of different acidic (A) and basic (B) residues on the stability of a peptide related to Marqusee and Baldwin's $i + 4 AB$. Gans et al. (1991) demonstrated, by using peptides comprised of only 8 Glu and

8 Lys residues, that sequence alone can determine whether or not a peptide forms a helix. These, and other studies, have demonstrated the importance of electrostatic interactions in the stability of some α -helical peptides. (The kinemage file on the Diskette Appendix illustrates side-chain ion pairs on helices in a native protein structure.) It is possible, however, to demonstrate α -helix formation in peptides that are not stabilized by specific side-chain interactions. Peptides that contain only alanine and either lysine (Marqusee et al., 1989) or glutamine (Scholtz et al., 1991b) residues are unable to make stabilizing side-chain interactions, nonetheless show stable helix formation mainly through making use of the high helix propensity of alanine.

In order to reach a more complete understanding of the role of electrostatic interactions in stabilizing α -helical peptides, we reopen the problem of the large differences in helix content among the four Glu-Lys and the two Asp-Lys peptides of Marqusee and Baldwin (1987, 1990).

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Table 1. Peptide sequences

Type ^a	Sequence	Length
Asp-Arg peptides		
<i>i</i> + 4 AB	Ac-A(DAAAR) ₃ Y-NH ₂	17
<i>i</i> + 3 AB	Ac-(ADAAR) ₃ Y-NH ₂	16
<i>i</i> + 4 BA	Ac-A(RAAAD) ₃ Y-NH ₂	17
<i>i</i> + 3 BA	Ac-(ARAAD) ₃ Y-NH ₂	16
Glu-Arg peptides		
<i>i</i> + 4 AB	Ac-A(EAAAR) ₃ Y-NH ₂	17
<i>i</i> + 3 AB	Ac-(AEAAR) ₃ Y-NH ₂	16
<i>i</i> + 4 BA	Ac-A(RAAAE) ₃ Y-NH ₂	17
<i>i</i> + 3 BA	Ac-(ARAAE) ₃ Y-NH ₂	16

^a A corresponds to either acidic residue (Asp or Glu) and B corresponds to the basic residue (Arg).

Their sequence design is used to examine the helical properties of Asp-Arg and Glu-Arg peptides. The sequences are given in Table 1. Our first aim is to compare the patterns of helix stabilization in these sets of peptides, to find out if different charged residue pairs give similar or different patterns. Our second aim is to find out if the effects of ion pair interactions can be separated from those of charge-helix dipole interactions by the combined use of pH titration and NaCl screening experiments. Our third aim is to find out if any differences in helix content among the four peptides remain at high ionic strength (5 M NaCl) and, if so, to investigate the interactions responsible for them.

Results

Properties of helix formation

These peptides show the same general properties of helix formation that have been observed previously (for review, see Scholtz & Baldwin, 1992). It should be noted, however, that Miick and coworkers (1992) have evidence suggesting that alanine-based peptides form mixed 3₁₀- and α -helical conformations. Peptides with these same general sequences have been used to investigate the helix-stabilizing properties of different ion pairs (Merutka & Stellwagen, 1991) and also to measure the relative helix propensities of all 20 amino acids (Merutka et al., 1990). Scholtz et al. (1991a) determined the parameters of helix-coil transition theory using peptides with an (AEAAKA)_{*n*} repeating sequence and chain lengths varying from 14 to 50 residues. All eight peptides studied here show measurable helix formation, based on the circular dichroism (CD) spectrum, at pH 7.0, 10 mM NaCl, 0 °C, except for *i* + 3 RD, which does, however, show partial helix formation at pH 2.5. The helix contents of all the peptides in this study are summarized in Table 2.

Table 2. Mean residue ellipticities of Asp-Arg and Glu-Arg peptides

Type ^a	- $[\theta]_{222}$ (deg cm ² dmol ⁻¹) ^b			
	0.01 M NaCl		1.0 M NaCl	
	pH 2.5	pH 7.0	pH 2.5	pH 7.0
Asp-Arg peptides				
<i>i</i> + 4 AB	15,800	25,200	15,000	21,500
<i>i</i> + 3 AB	11,100	16,300	10,300	12,600
<i>i</i> + 4 BA	11,700	14,000	14,300	14,800
<i>i</i> + 3 BA	4,900	2,200	7,400	5,100
Glu-Arg peptides				
<i>i</i> + 4 AB	27,400	31,300	28,000	29,200
<i>i</i> + 3 AB	15,700	22,400	16,400	19,500
<i>i</i> + 4 BA	24,900	22,500	27,900	23,800
<i>i</i> + 3 BA	18,000	11,800	23,000	16,900

^a A corresponds to either acidic residue (Asp or Glu) and B corresponds to the basic residue (Arg).

^b Mean residue ellipticity at 222 nm. Error in $-\theta_{222}$ is ± 500 (deg cm² dmol⁻¹). The fractional helicity can be determined from $-\theta_{222}$ (Scholtz et al., 1991a).

We tested for helix stabilization caused by peptide association in two ways. The thermal unfolding curves of peptides *i* + 4 DR and *i* + 4 ER were measured at two peptide concentrations that differed by 10-fold and the concentration dependence of $[\theta]_{222}$ was measured from 0 to 200 μ M, both at 0 and 25 °C, for the other six peptides. No dependence on peptide concentration was found in either set of experiments, indicating that the peptides appear to show monomeric helix formation. The molecular weight of a related alanine-based peptide was measured using sedimentation equilibrium by Padmanabhan et al. (1990), who found that it was monomeric.

Dependence of helix content on pH

Figure 1 shows the pH dependence of helix content, measured at 0 °C in 10 mM NaCl, for both the Asp-Arg and Glu-Arg peptides. The AB peptides in both sets show a drop in helix content in going from pH 7 to pH 2, caused by the removal of favorable ion pair and charge-dipole interactions upon protonation of the Asp or Glu residues. Surprisingly, three out of four BA peptides show an increase in helix content from pH 7 to pH 2. This suggests that the effect of unfavorable charge-helix dipole interactions in these peptides outweighs the stabilizing effect of ion pair interactions at pH 7. Because ion pair and acidic charge-helix dipole interactions are broken at pH 2, the remaining observed changes in helix content are due to charge-helix dipole interactions involving Arg residues and possibly the changes in helix propensity, or *s*-value, of Glu and Asp that occur on protonation.

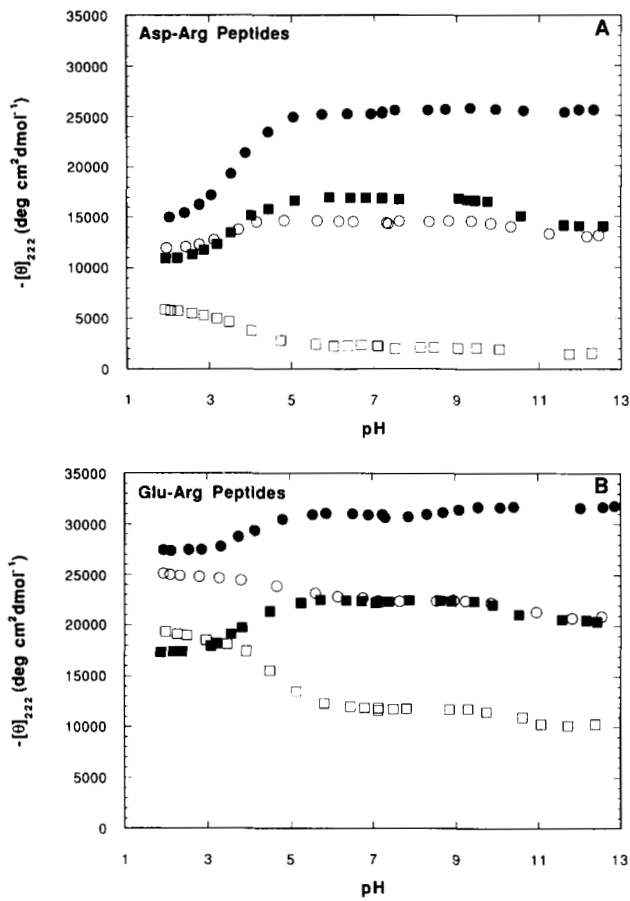


Fig. 1. The pH titrations of helix content in 10 mM NaCl at 0 °C. **A:** Asp-Arg peptides. **B:** Glu-Arg peptides. ●, *i* + 4 AB; ○, *i* + 4 BA; ■, *i* + 3 AB; □, *i* + 3 BA. $[\theta]_{222}$ is the mean residue ellipticity at 222 nm.

Dependence of helix content on NaCl concentration

Figure 2 shows, for all eight peptides, the change in helix content caused by screening charge interactions at pH 7.0 with NaCl. Ion pair interactions should be screened effectively by 1 M NaCl, whereas charge-helix dipole interactions are screened more slowly (see Discussion). All four BA peptides show increases in helix content between 0 and 1 M NaCl. The helix content of the *i* + 3 BA peptides rises markedly between 0 and 1 M NaCl because helix-destabilizing charge interactions, as well as the intrinsic destabilizing effect of helix macrodipole, can be screened by NaCl. The evidence for screening the destabilizing helix macrodipole with NaCl is shown in a completely neutral peptide helix (Scholtz et al., 1991b). The four AB peptides show either downward curvature, indicative of screening helix-stabilizing interactions, or else the screening curves are nearly straight, suggesting that screening favorable charge interactions is roughly compensated by screening the unfavorable helix macrodipole. All peptides show a decrease in helix content with

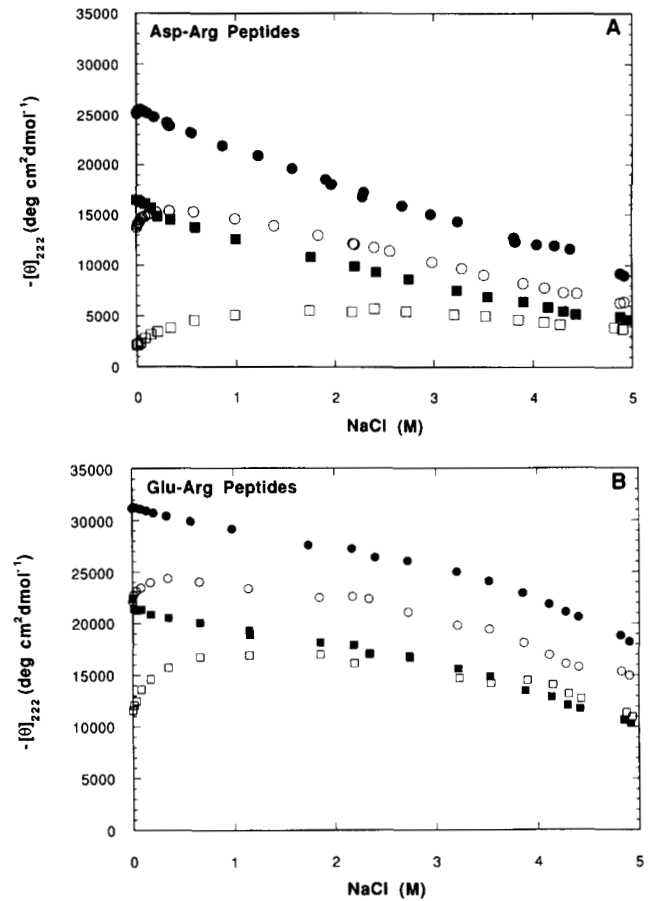


Fig. 2. Screening by NaCl of charge interactions that affect helix content at pH 7.0, 0 °C. **A:** Asp-Arg peptides. **B:** Glu-Arg peptides. ●, *i* + 4 AB; ○, *i* + 4 BA; ■, *i* + 3 AB; □, *i* + 3 BA.

NaCl concentration above 2 M: this is the Hofmeister effect, which is linear in NaCl concentration when ΔG^0 , the difference in Gibbs energy between helix and coil, is plotted against the ionic strength of the solution (see Discussion). A notable feature of these curves is the fact that the helix-stabilizing (or destabilizing) interactions are not completely screened by 5 M NaCl, and striking differences in helix content remain.

At pH 2.5, the screening effects of NaCl are reduced in magnitude (Fig. 3) but are still present, and the difference in behavior between BA and AB peptides remains qualitatively the same as at pH 7.0. This clearly implicates Arg charge-helix dipole interactions as a major cause of these differences, because ion pair interactions are broken at pH 2.5, and the only remaining charge interactions are the ones involving Arg residues and the helix dipole. At pH 2.5, as at pH 7.0, important differences in helix content among the four peptides of each set remain at 5 M NaCl. This effect is more striking for the Glu-Arg (Fig. 3B) than for the Asp-Arg peptides (Fig. 3A).

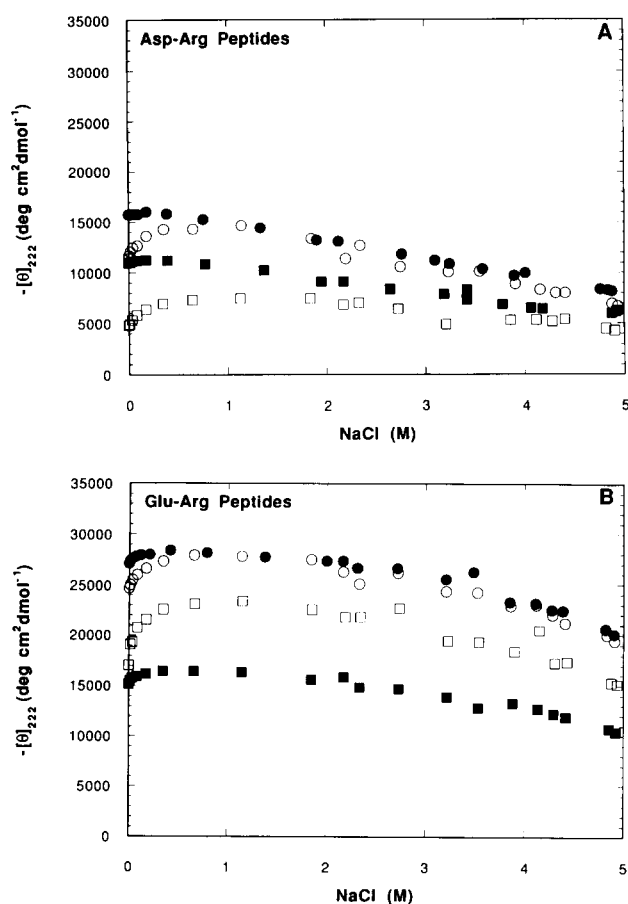


Fig. 3. NaCl screening of charge interactions at pH 2.5, 0 °C. A: Asp-Arg peptides. B: Glu-Arg peptides. ●, $i + 4$ AB; ○, $i + 4$ BA; ■, $i + 3$ AB; □, $i + 3$ BA.

Discussion

Pattern of helix stabilization in Asp-Arg and Glu-Arg peptides

The notable result from these experiments is that large differences in helix content at pH 7.0 are found among the four peptides of the Asp-Arg and Glu-Arg sets in this study as well as the Glu-Lys and Asp-Lys peptide sets studied previously (Marqusee & Baldwin, 1987, 1990). The pattern is: $i + 4$ AB $>$ $i + 4$ BA \approx $i + 3$ AB $>$ $i + 3$ BA. Consequently, these helical properties are intrinsic features of the sequence design and are nearly independent of the chemical types of residue. At pH 2.5 the pattern of helix stabilization is also almost the same for each set of peptides as it is at pH 7.0. The exception involves peptides $i + 3$ ER and $i + 3$ RE, the latter being more helical at all NaCl concentrations at pH 2.5.

It is important now to characterize the properties of single ion pairs and the interactions of single charged residues with the helix dipole. The availability of a neutral

Gln-Ala peptide helix (Scholtz et al., 1991b) suggests that such studies are feasible. It is also important to establish which side-chain rotamers are capable of forming strong ion pair interactions, for a given pair of oppositely charged residues, and which rotamers interact best with the helix dipole. Perutz and Fermi (1988) discuss possible reasons for the Glu-Lys results of Marqusee and Baldwin (1987). They emphasize the possible roles of helix-stabilizing van der Waals contacts between side-chain rotamers and the alanine helix when H-bonded ion pairs (salt bridges) are formed. They also suggest that the presence of $i, i + 2$ spacings between oppositely charged residues in the $i + 3$ AB and $i + 3$ BA peptides may tend to destabilize these helices. The peptides with oppositely charged residues spaced $i, i + 2$ apart may form ion pairing hairpin loops, which push the helix-coil equilibrium toward the coil.

The helix content of each of the Asp-Arg peptides is lower than the helix content for the analogous Glu-Arg peptides. This may indicate that Asp has a lower helix-forming tendency than Glu, as has been observed in other model peptide systems (Merutka et al., 1990; O'Neil & DeGrado, 1990; Wojcik et al., 1990), or that the Glu-containing peptides are able to form better stabilizing side-chain interactions than the corresponding Asp peptides. On the other hand, comparing the Arg peptides with the analogous Lys peptides characterized by Marqusee and Baldwin (1987, 1990), smaller differences in helix content are seen. A more complete understanding of these differences will be realized from the studies of single ion pairs and isolated charged residues in the neutral Ala-Gln peptide helix (Scholtz et al., 1991b).

Separation of ion pair and charge-helix dipole interactions

We were unable to find any satisfactory way of separating ion pair from charge-helix dipole interactions. Both types of interactions are broken by acidic titration; ion pair and acidic charge-dipole interactions are broken completely, whereas the interactions of charged Arg side chains with the helix dipole remain at low pH. Any change in helix propensity caused by protonating Glu or Asp residues also will contribute to the change in helix content between pH 7 and pH 2. Ion pair interactions are screened much more rapidly by NaCl than are charge-helix dipole interactions, but it is difficult to separate the two classes by this property because of the added presence of the Hofmeister effect at high NaCl concentration and of the screenable helix-destabilizing effect of the intrinsic helix macrodipole at low NaCl concentration.

Although it is difficult to separate the effects of ion pair interactions and charge-dipole interactions from an inspection of the effect of NaCl on the observed helicity, it does appear that ion pair interactions are screened more efficiently by NaCl than charge-dipole interactions. A discussion of counterion screening in terms of the Debye-

Hückel theory is given by Cantor and Schimmel (1980). They point out that the Debye–Hückel screening parameter κ has the dimensions of reciprocal length, and that the interaction between two fixed charges is screened effectively when $(1/\kappa)$ drops below the distance between the two charges. When the charge separation is 3 Å, this condition is met at 1 M NaCl.

Residual differences in helix content within each set at high ionic strength

Substantial differences in helix content remain at 5 M NaCl among the four peptides of each set, both at pH 7.0 and pH 2.5 (see Figs. 2, 3). The question then arises whether these differences are caused by nonscreenable interactions (e.g., H-bond interactions) or by charge–helix dipole interactions that are incompletely screened by 5 M NaCl. There is a straightforward test to decide between these explanations: if the cause is incomplete screening, then the screening curves for the four peptides should converge to a common value at some higher NaCl concentration, when plotted appropriately. The Hofmeister effect is linear when ΔG^0 , the difference in Gibbs energy between helix and random coil, is plotted against M NaCl (Scholtz et al., 1991b). Because calculating ΔG^0 introduces new parameters, and any error associated with those parameters, it seems preferable to determine experimentally if the residual screening or H-bonding effect goes to zero at much higher salt concentrations, by using a salt that is more water soluble than NaCl. This approach is left to future work. Ihara et al. (1982) present interesting data on the screening of charge–helix dipole interactions by various chlorides and by potassium fluoride. They give data on the helical stability of a 20-residue alanine peptide with a block of 20 ionized glutamate residues either at the N-terminal end (EAF) or at the C-terminal end (AEF) of the alanine helix. They show that the helix formed by EAF is strikingly more stable than that of AEF (ΔT_m is more than 40 °C at 0.01 M NaCl), and that the T_m values are converging at high chloride concentrations. From their data, one may conclude that charge–helix dipole interactions are screened only slowly with increasing NaCl concentration, and that screening should approach completion near 5 M NaCl or possibly at some higher concentration.

Our results clearly indicate that significant differences in helix content among these peptides, which differ only in orientation and spacing of the charged groups, remain at high ionic strength. The nature of these differences may be the result of nonscreenable H-bond interactions or remaining charge–dipole effects. It seems plausible that incomplete screening of charge–helix dipole interactions is the best explanation for the differences in helix content at 5 M NaCl, but further work is needed to resolve this problem.

Materials and methods

Peptide synthesis and purification

Peptides were synthesized by the solid-phase method using an active ester coupling procedure, employing pentafluorophenyl esters of 9-fluorenylmethoxy-carbonyl amino acids (Atherton & Sheppard, 1985). Peptides were cleaved from the resin and deprotected by using an N₂-sparged mixture of 2% anisole, 3% ethanedithiol, 5% thioanisole, and 90% trifluoroacetic acid. Arg deprotection was monitored by fast-performance liquid chromatography (FPLC). The cleavage reaction was allowed to proceed until a single deprotected peak was observed by FPLC. Peptides were purified as described by Chakrabarty et al. (1991). Peptide purity was assessed by FPLC analysis (Pharmacia) and by capillary electrophoresis (Applied Biosystems); purity was greater than 95% for each peptide. The primary-ion molecular weight of each peptide was confirmed by fast atom bombardment mass spectrometry.

Spectral measurements

CD measurements were made on an AVIV 60 DS spectropolarimeter equipped with a Hewlett-Packard model 89100A temperature controller. The ellipticity is reported as mean residue ellipticity, $[\theta]$, and was calibrated with (+)-10-camphorsulfonic acid. Cuvettes with 1-cm and 1-mm pathlengths were used. The degree of helical structure in each peptide was determined by monitoring the ellipticity at 222 nm.

Concentrations of the peptide stock solutions were determined by measuring Tyr absorbance in 6 M guanidine hydrochloride with 20 mM phosphate buffer ($\epsilon_{275} = 1,450 \text{ M}^{-1} \text{ cm}^{-1}$) (Brandts & Kaplan, 1973). Samples were prepared for CD analysis at 222 nm by diluting the stock solution with a buffer consisting of 1 mM sodium citrate, 1 mM sodium borate, and 1 mM sodium phosphate (CD buffer). Samples for spectral analysis were prepared in 10 mM potassium fluoride and 1 mM potassium phosphate. The pH titrations were performed by lowering the pH with aliquots of concentrated HCl for half the sample and by raising the pH of the other half with NaOH. Measurements of pH were made on an Orion pH meter model 601 A using a Metrohm electrode and calibrated at 0 °C by adjusting the standard solutions to manufacturer's specification. NaCl dependences were measured by adding incremental volumes of 5 M NaCl in CD buffer to a solution of peptide in CD buffer with no salt.

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References

- Atherton, E. & Sheppard, R.C. (1985). Solid phase peptide synthesis using N-alpha-fluorenyl-methoxycarbonylamino acid pentafluorophenyl esters. *J. Chem. Soc. Chem. Commun.* 3, 165-166.
- Brandts, J.R. & Kaplan, K.J. (1973). Derivative spectroscopy applied to tyrosyl chromophores. Studies on ribonuclease, lima bean inhibitor, insulin, and pancreatic trypsin inhibitor. *Biochemistry* 12, 2011-2024.
- Cantor, C.R. & Schimmel, P.R. (1980). *Biophysical Chemistry*, Vol. III: *The Behavior of Biological Macromolecules*, p. 1115. W.H. Freeman and Company, New York.
- Chakrabarty, A., Schellman, J.A., & Baldwin, R.L. (1991). Large differences in the helix propensities of alanine and glycine. *Nature* 351, 586-588.
- Gans, P.J., Lyu, P.C., Manning, M.C., Woody, R.W., & Kallenbach, N.R. (1991). The helix-coil transition in heterogeneous peptides with specific side-chain interactions: Theory and comparison with CD spectral data. *Biopolymers* 31, 1605-1614.
- Ihara, S., Ooi, T., & Takahashi, S. (1982). Effects of salts on the non-equivalent stability of the α -helices of isomeric block copolypeptides. *Biopolymers* 21, 131-145.
- Marqusee, S. & Baldwin, R.L. (1987). Helix stabilization by Glu⁻...Lys⁺ salt bridges in short peptides of *de novo* design. *Proc. Natl. Acad. Sci. USA* 84, 8898-8902.
- Marqusee, S. & Baldwin, R.L. (1990). α -Helix formation by short peptides in water. In *Protein Folding* (Gierasch, L.M. & King, J., Eds.), pp. 85-94. American Association for the Advancement of Science, Washington, D.C.
- Marqusee, S., Robbins, V.H., & Baldwin, R.L. (1989). Unusually stable helix formation in short alanine-based peptides. *Proc. Natl. Acad. Sci. USA* 86, 5286-5290.
- Merutka, G., Lipton, W., Shalongo, W., Park, S., & Stellwagen, E. (1990). Effect of central-residue replacement on the helical stability of a monomeric peptide. *Biochemistry* 29, 7511-7515.
- Merutka, G. & Stellwagen, E. (1991). Effect of amino acid ion pairs on peptide helicity. *Biochemistry* 30, 1591-1594.
- Miick, S.M., Martinez, G.V., Fiori, W.R., Todd, A.P., & Millhauser, G.L. (1992). Short alanine-based peptides may form 3_{10} -helices in solution and not α -helices. *Nature* 359, 653-655.
- O'Neil, K.T. & DeGrado, W.F. (1990). A thermodynamic scale for the helix-forming tendencies of the commonly occurring amino acids. *Science* 250, 646-651.
- Padmanabhan, S., Marqusee, S., Ridgeway, T., Laue, T.M., & Baldwin, R.L. (1990). Relative helix-forming tendencies of nonpolar amino acids. *Nature* 344, 268-270.
- Perutz, M.F. & Fermi, G. (1988). Stereochemistry of salt-bridge formation in α -helices and β -strands. *Proteins Struct. Funct. Genet.* 4, 294-295.
- Scholtz, J.M. & Baldwin, R.L. (1992). The mechanism of α -helix formation in peptides. *Annu. Rev. Biophys. Biomol. Struct.* 21, 95-118.
- Scholtz, J.M., Qian, H., York, E.J., Stewart, J.M., & Baldwin, R.L. (1991a). Parameters of helix-coil transition theory for alanine-based peptides of varying chain lengths in water. *Biopolymers* 31, 1463-1470.
- Scholtz, J.M., York, E.J., Stewart, J.M., & Baldwin, R.L. (1991b). A neutral, water-soluble α -helical peptide: The effect of ionic strength on the helix-coil equilibrium. *J. Am. Chem. Soc.* 113, 5102-5104.
- Wojcik, J., Altman, K.H., & Scheraga, H.A. (1990). Helix-coil stability constants for the naturally occurring amino acids in water. XXIV. Half-cystine parameters from random poly(hydroxybutyl)glutamine-co-S-methylthio-L-cysteine). *Biopolymers* 30, 121-134.