Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 8898–8902, December 1987
Biochemistry

Helix stabilization by $Glu^{-}\cdots Lys^{+}$ salt bridges in short peptides of *de novo* design

(model α-helix/protein folding/protein electrostatics)

SUSAN MARQUSEE AND ROBERT L. BALDWIN

Department of Biochemistry, Stanford University Medical Center, Stanford, CA 94305

Contributed by Robert L. Baldwin, August 31, 1987

ABSTRACT **Four alanine-based peptides were designed, synthesized, and tested by circular dichroism for α-helix formation in H_2O. Each peptide has three glutamic/lysine residue pairs, is 16 or 17 amino acids long, and has blocked α-NH_2 and α-COOH groups. In one set of peptides ("*i*+4"), the glutamic and lysine residues are spaced 4 residues or 1 residue apart. In the other set ("*i*+3"), the spacing is 3 or 2 residues. Within each of these sets, a pair of peptides was made in which the positions of the glutamic and lysine residues are reversed [Glu, Lys (E,K) vs. Lys, Glu (K,E)] in order to assess the interaction of the charged side chains with the helix dipole. Since the amino acid compositions of these peptides differ at most by a single alanine residue, differences in helicity are caused chiefly by the spacing and positions of the charged residues. The basic aim of this study was to test for helix stabilization by (Glu^{-}, Lys^{+}) ion pairs or salt bridges (H-bonded ion pairs). The results are as follows. (*i*) All four peptides show significant helix formation, and the stability of the α-helix does not depend on peptide concentration in the range studied. The best helix-former is (*i*+4)E,K, which shows ≈80% helicity in 0.01 M NaCl at pH 7 and 0°C. (*ii*) The two *i*+4 peptides show more helix formation than the *i*+3 peptides. pH titration gives no evidence for helix stabilization by *i*+3 ion pairs. (*iii*) Surprisingly, the *i*+4 peptides form more stable helices than the *i*+3 peptides at extremes of pH (pH 2 and pH 12) as well as at pH 7. These results may be explained by helix stabilization through $Glu^{-}\cdots Lys^{+}$ salt bridges at pH 7 and singly charged H bonds at pH 2 ($Glu^{0}\cdots Lys^{+}$) and pH 12 ($Glu^{-}\cdots Lys^{0}$). The reason why these links stabilize the α-helix more effectively in the *i*+4 than in the *i*+3 peptides is not known. (*iv*) Reversal of the positions of glutamic and lysine residues usually affects helix stability in the manner expected for interaction of these charged groups with the helix dipole. (*v*) α-Helix formation in these alanine-based peptides is enthalpy-driven, as is helix formation by the C-peptide of ribonuclease A.**

It is important to find out whether the α-helix can be formed in H_2O by short peptides that have simple repetitive sequences. Such helix-forming peptides could provide a well-defined system for detecting and measuring specific side-chain interactions. Pioneering studies with this goal were initiated by Sela, Katchalski-Katzir, and coworkers (1–3). One basic problem is that short peptides (≤20 amino acids) are predicted not to show observable helix formation in aqueous solution (4, 5) when the Zimm–Bragg equation (6) is used with parameters (s and σ) determined by "host–guest" experiments. Nevertheless, the 13-residue C-peptide (residues 1–13) obtained from RNase A does show measurable helicity (≈25%) at low temperatures (4, 7); moreover, residue substitutions have been found that substantially enhance its helicity (8). Specific side-chain interactions, factors that are not considered in the Zimm–Bragg model, are responsible at least in part for the fact that the C-peptide is much more helical than predicted (4, 5, 8–10).

A second basic problem in studying α-helix formation by short peptides in H_2O is that most residues designated as good helix-formers [either by host–guest studies (11) or by frequency in protein helices (12)] are hydrophobic residues, and peptides made from them are insoluble in H_2O. Alanine would provide a good foundation for designed peptides because its small $-CH_3$ side chain should not interfere with side-chain interactions between other residues. In this work we ask whether alanine-based peptides can be solubilized by inserting specific pairs of lysine and glutamic residues, whether such peptides will form the α-helix in H_2O, and whether the helix formed is stabilized by specific ($Glu^{-}\cdots Lys^{+}$) salt bridges or ion pairs.

Certain problems must be considered in the design of helix-forming peptides. First, short peptides often form the α-helix in H_2O only because the helix is amphipathic and is stabilized by peptide aggregation along the hydrophobic face. In order to avoid this, the peptide can be designed so that the charged residues spiral around the helix. If the equilibrium between helix and random coil is freely mobile, one can check directly for aggregation-induced helix formation by testing whether helix stability is concentration-dependent or by using a sizing column to measure the molecular size of the helix-forming unit (8).

A second factor to consider in the design of these peptides is the specific placement of the interacting residues. To avoid ambiguity in interpreting the experimental results, fixing the residue spacing between these side chains is critical. Fig. 1 shows the sequences of the peptides studied here. In one pair of peptides ("*i*+4"), Glu^{-} (E) and Lys^{+} (K) residues are positioned at alternating spacings of *i*+4 and *i*+1 residues. In a second pair ("*i*+3"), the spacing alternates between *i*+3 and *i*+2.

Interaction of charged residues with the α-helix dipole can either stabilize or destabilize the helix (5, 8) and must be taken into account in the design of these experiments. The α-NH_3^{+} and α-COO^{-} groups of the peptides are blocked with acetyl and amide groups, respectively (see Fig. 1), to avoid the helix-destabilizing interactions of these groups with the helix dipole. Each charged residue may interact with the helix dipole (13), and the sign of this interaction can be reversed by reversing the charge. Consequently, the peptides studied here are made in pairs and the positions of the Glu^{-} and Lys^{+} residues are interchanged in one member of each pair. Since the positive pole of the α-helix dipole is near the N terminus and the negative pole is near the C terminus, the helix dipole interactions should be favorable when there is a Glu^{-} residue close to the N terminus and a Lys^{+} residue close to the C terminus. This is denoted as the E,K orientation, whereas K,E refers to the reverse orientation.

Our goal was to detect ion pairs or salt bridges that increase helix stability. Oppositely charged residues that are appro-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

(i+4) E,K: Ac A E A A A K E A A A K E A A A K A NH_2
(i+4) K,E: Ac A K A A A E K A A A E K A A A E A NH_2 17mer

(i+3) E,K: Ac A E A A K A E A A K A E A A K A NH_2
(i+3) K,E: Ac A K A A E A K A A E A K A A E A NH_2 16mer

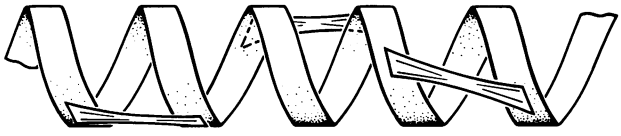

FIG. 1. (*Upper*) Sequences of the four peptides designed. Ac, acetyl; A, alanine; E, glutamic acid; K, lysine. (*Lower*) Diagram illustrates the potential helix-anchoring effect caused by salt bridges or ion pairs for the $i+4$ peptides. Note that the charged groups spiral around the surface of the helix.

priately spaced across a single turn of the helix could nucleate the helix by stabilizing one turn and thus anchoring the helical conformation. The diagram in Fig. 1 illustrates a helix stabilized by three such interacting pairs of (Glu^-, Lys^+) residues. We use the term "ion pair" here to denote a simple nonbonded interaction between oppositely charged ions; the term "salt bridge" refers to a H-bonded ion pair. Our experiments do not distinguish directly between salt bridges and ion pairs, but the results of pH-titration experiments suggest that salt bridges do form. To test for charged side-chain interactions that increase helix stability, we compared the helix content in peptides of nearly identical lengths and amino acid composition but differing in the spacing between charged residues.

MATERIALS AND METHODS

Peptide Synthesis. Peptides were synthesized by the solid-phase method (14), using methylene chloride as the solvent and 33% trifluoroacetic acid/1% indole/methylene chloride for deprotection. Couplings were monitored by the Kaiser test and repeated until complete or capped with acetic anhydride. The peptides were synthesized as C-terminal amides on *p*-methylbenzhydrylamine (polystyrene/1% divinylbenzene) resin. The N termini were acetylated by using acetic anhydride with an equivalent of triethylamine in dimethylformamide. A 10-fold excess of acetylating agent was used and the reaction went to completion within 30 min.

Peptide Purification. Peptides were purified by reverse-phase chromatography on C_{18} resin, using a gradient of 5–60% acetonitrile in 0.1% trifluoroacetic acid, and by gel filtration on Sephadex G-25 in 10 mM HCl or 10 mM acetate (pH 4.5). Peptide purity and amino acid composition were determined by FPLC (Pharmacia), ^1H NMR, and amino acid analysis. The primary ion molecular weight was determined by fast-atom bombardment mass spectrometry.

Circular Dichroism (CD). Peptide concentrations of ≈2 mM stock solutions in H_2O were determined by the ninhydrin method, using leucine as a standard (15). The reproducibility of the peptide concentration determinations was ±5%. CD samples were prepared by diluting stock solutions with a 1 mM sodium citrate/1 mM sodium borate/1 mM sodium phosphate buffer of the indicated NaCl concentration. In order to obtain spectra below 200 nm, some samples were prepared in unbuffered 10 mM KF. The pH was adjusted with HCl and NaOH. CD spectra were taken routinely at peptide concentrations around 15 μM.

CD measurements were made on a modified Cary 60 spectropolarimeter (model 60DS, AVIV, Lakewood, NJ), either at Stanford University or in the laboratory of P. S. Kim (Whitehead Institute), and a Jasco J500A spectropolarimeter in the laboratory of J. T. Yang (San Francisco Medical School, University of California). Cuvettes with 10-mm, 5-mm, and 1-mm pathlengths were used. Ellipticity is reported as mean residue ellipticity, [θ], and was calibrated with (+)-10-camphorsulfonic acid (16).

RESULTS

Properties of Helix Formation. Peptide helix formation was monitored by CD. At low temperature (1°C) and at pH 7, all four peptides show significant helix content. The CD spectra are characteristic of an α-helix (17) (Fig. 2): each shows a distinct minimum at 222 nm (n–π^* transition) and a second minimum close to 208 nm (a superposition of the random coil π–π^* transition at 200 nm and the α-helix π–π^* transition at 208 nm) followed by the α-helix maximum at 193 nm. Fig. 2 illustrates sample spectra for the peptide ($i+4$)E,K. Helical content is most easily monitored by following the intensity of the minimum at 222 nm ($-[\theta]_{222}$).

Helix formation by these peptides is a monomolecular process and is not the result of aggregation. All spectra are independent of concentration in the measured range of 5–80 μM. Fig. 3*b* shows examples taken with the peptides ($i+4$)E,K and ($i+3$)K,E. A sizing-column experiment, using Sephadex G-25 Superfine (8), showed that peptide ($i+4$)E,K (17 residues) is eluted between C-peptide carboxylate (13 residues) and S-peptide (19 residues) in optimal helix-forming conditions (pH 7.0, 0°C, 0.01 M NaCl), as expected if these peptides are monomeric.

The helical structure in these peptides is strongly dependent on temperature. Figs. 2 and 3*a* show that helix formation is enthalpy-driven, with maximal helicity at low temperature; unfolding occurs with increasing temperature. This behavior is consistent with previous studies of C-peptide analogs. The same type of thermal unfolding is observed for the best helix-former, ($i+4$)E,K, as for the poorest helix-former, ($i+3$)K,E (Fig. 3*a*). The thermal transition is very broad: at 70°C, the spectra indicate that a small fraction of helix still persists, and the failure to reach a plateau at low temperature implies that the transition to 100% helix formation is not complete at 0°C. CD spectra taken through the transition (Fig. 2) show an isodichroic point at 202 nm, which is consistent with a two-state transition with each residue being in either a helical or random-coil conformation. In summary, these data demonstrate that the peptides undergo a broad

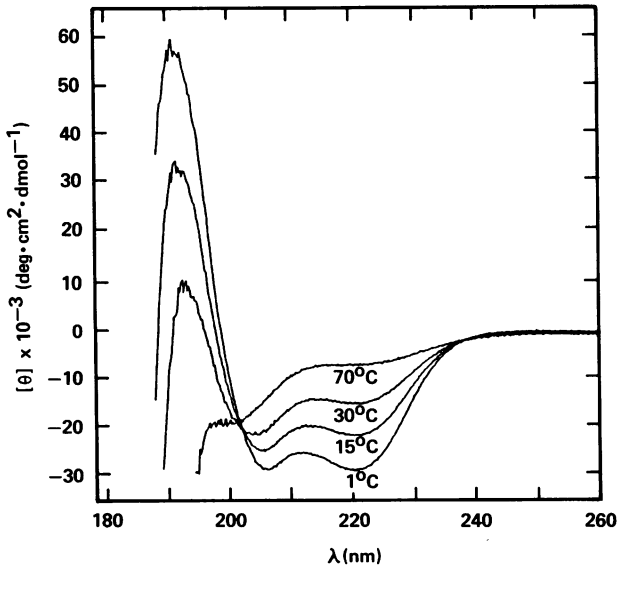

FIG. 2. CD spectra of peptide ($i+4$)E,K (17 μM) at four temperatures in 0.01 M KF (pH 7.0). deg, Degree.

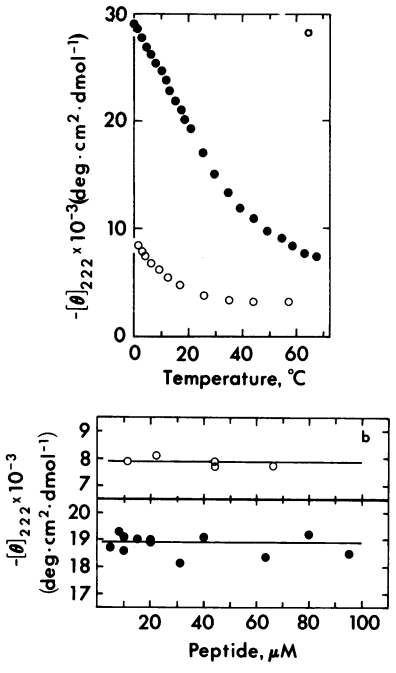

FIG. 3. (*a*) Thermal unfolding profiles for two peptides measured by $-[\theta]_{222}$, the mean residue ellipticity at 222 nm. ●, (*i*+4)E,K in 0.01 M NaCl (pH 7.3); ○, (*i*+3)K,E in 0.01 M NaCl (pH 7.0). (*b*) A test for dependence of helicity on peptide concentration. ●, (*i*+4)E,K in 0.01 M NaCl (pH 7.1) at 20°C; ○, (*i*+3)K,E in 0.01 M NaCl (pH 7.3) at 1°C.

transition from the random coil to a unique structure, the α-helix, induced by a decrease in temperature.

Table 1 lists the extent of helix formation (measured by $-[\theta]_{222}$) for all four peptides at pH 7 and at 0.01 and 1.0 M NaCl. Both the glutamic and the lysine residues are ionized at this pH. Under these conditions, the average helical content is clearly different in each peptide: at 0.01 M NaCl, $-[\theta]_{222}$ ranges from 29,000 degree·cm^2·dmol^{-1} for (*i*+4)E,K to 8500 for (*i*+3)K,E. Since the peptides have almost identical amino acid compositions, this confirms the importance of amino acid sequence, and hence side-chain interactions, in helix stability.

Reversal of Lysine and Glutamic Residues. Effects caused by reversing the charge orientation (E,K or K,E) are larger in the *i*+3 peptides than in the *i*+4 peptides and are greater at 0.01 M than at 1.0 M NaCl (Table 1). For the *i*+3 peptides, the change in $-[\theta]_{222}$ for E,K→K,E is very striking: from 17,600 to 8500 at 0.01 M NaCl and from 17,400 to 12,000 at 1.0 M NaCl. These changes agree with expectation for a helix dipole interaction: in the E,K peptides this interaction is expected to stabilize the helix, whereas in the K,E peptides it should be helix-destabilizing. For the *i*+4 peptides, the change is less dramatic: $-[\theta]_{222}$ drops from 29,000 to 25,300 at 0.01 M NaCl for E,K→K,E, but at 1.0 M NaCl the change is within the experimental error.

***i*+4 Versus *i*+3 Residue Spacing (pH 7).** A comparison of helix content between peptides with different charge spacings (*i*+4 or *i*+3) and the same orientation (E,K or K,E) should reveal any differential stabilization caused by particular residue spacings. At pH 7 and 0.01 M NaCl, where helix stabilization by salt bridges or ion pairs should be optimal, the value of $-[\theta]_{222}$ is about 11,000 greater in (*i*+4)E,K than in (*i*+3)E,K. Similarly, in the K,E orientation, $-[\theta]_{222}$ of (*i*+4)K,E is 17,000 greater than that of (*i*+3)K,E. Therefore, in both E,K and K,E orientations, salt bridges or ion pairs formed by the *i*+4 peptides stabilize the helical conformation relative to the *i*+3 peptides.

Table 1. Helix content (pH 7.0, 1°C)

Peptide	$-[\theta]_{222}$, degree·cm^2·dmol^{-1}		Helix dipole interaction
	0.01 M NaCl	1.0 M NaCl	
(*i*+4)E,K	29,000	24,800	+
(*i*+4)K,E	25,300	25,700	−
(*i*+3)E,K	17,600	17,400	+
(*i*+3)K,E	8,500	12,000	−

pH and NaCl Dependences. In order to investigate the specific nature of the stabilizing interactions within each peptide, CD measurements are made at different states of side-chain ionization. Fig. 4 illustrates this pH titration of helix content as monitored by $-[\theta]_{222}$. At low ionic strength (0.01 M NaCl, 1°C), the curve for (*i*+4)E,K is bell-shaped (Fig. 4*a*). At both pH extremes, $-[\theta]_{222}$ is ≈26,000, and it increases to ≈29,000 at neutral pH. The apparent pK values are ≈4 and ≈10.5. These pK values are assigned to the titration of glutamic and lysine residues, respectively. It is evident that titration of these residues does affect helix formation. The pH dependence of helix content in (*i*+4)K,E (Fig. 4*b*) is similar. In both *i*+4 peptides the helices are most stable at neutral pH, where glutamic and lysine residues are ionized.

The pH titrations of helix content for the *i*+3 peptides are very different from those of the *i*+4 peptides. Fig. 4*d* shows that the pH dependence for (*i*+3)K,E is an inverted bell-shaped curve. Unlike the *i*+4 peptides, this peptide is least stable at neutral pH ($-[\theta]_{222}$ = 8,500). The pH dependence of helix stability changes dramatically in the *i*+3 peptides when the (Glu^-, Lys^+) direction is reversed, in contrast to the *i*+4 peptides. In (*i*+3)E,K, helix content is higher at neutral pH than at pH 2 (Fig. 4*c*).

The NaCl dependence of helix content differs markedly from one peptide to the next and also varies with pH for a given peptide (Table 1, Fig. 5).

DISCUSSION

Helix Formation by Alanine-Based Peptides of Designed Sequence. Three conditions must be satisfied for this peptide system to yield useful information about Glu–Lys side-chain interactions in α-helices. (*i*) The peptides must be soluble

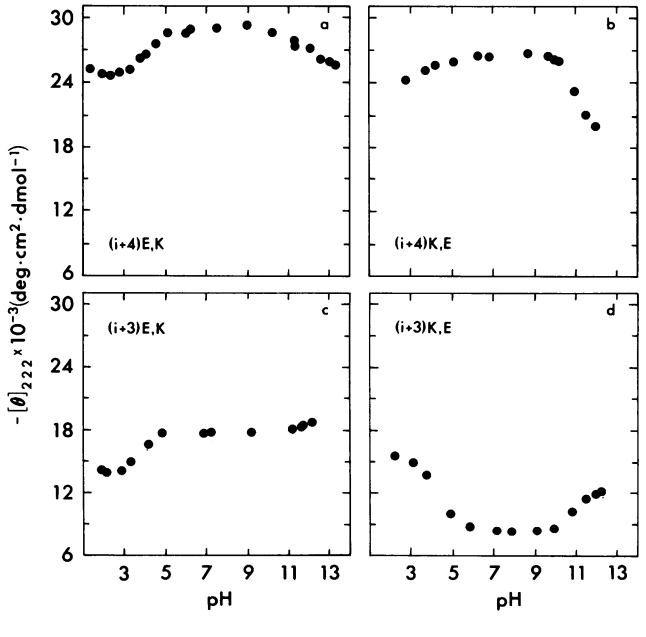

FIG. 4. pH titration of helicity for the four peptides in 0.01 M NaCl at 1°C. (*a*) (*i*+4)E,K. (*b*) (*i*+4)K,E. (*c*) (*i*+3)E,K. (*d*) (*i*+3)K,E.

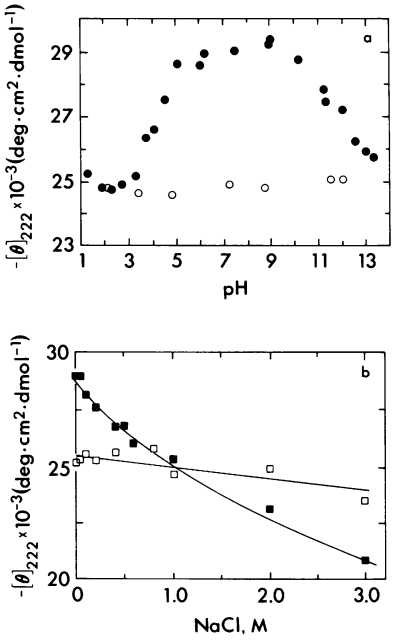

FIG. 5. Dependence of helicity on NaCl concentration for peptide (i+4)E,K. (*a*) pH titration at 1°C in 0.01 M (●) and 1.0 M (○) NaCl. (*b*) NaCl dependence (1°C) at pH 7.1 (■) and at pH 2.25 (□).

(oligoalanine peptides are insoluble in H_2O). (*ii*) Some of the peptides must show measurable α-helix formation. (*iii*) The α-helix must be formed in a monomolecular reaction.

Our results show that these conditions are satisfied with surprising ease. The peptides dissolve easily and there is no problem in making solutions in the concentration range used for CD studies (10–100 μM). Evidently, the ionized Glu^- and Lys^+ side chains are effective solubilizing agents. The peptides also show surprisingly high helix contents. Table 1 shows that the best helix-former, (i+4)E,K, contains ≈80% helix in 0.01 M NaCl at 1°C (based on $-[\theta]_{222}$ = 36,000 for 100% helix in trifluoroethanol/H_2O mixtures; S.M., unpublished data) and even the poorest helix-former, (i+3)K,E, shows about 25% helix content in 0.01 M NaCl. Finally, the helices are not stabilized by aggregation, since $-[\theta]_{222}$ is independent of peptide concentration and (i+4)E,K is eluted at the position expected for a monomer on a Sephadex G-25 sizing column.

Helix Stabilization by Salt Bridges and Singly Charged H Bonds in the i+4 Peptides. The data shown in Table 1 indicate that the i+4 peptides are stabilized by ion pairs or salt bridges relative to the i+3 peptides. The reasons for this behavior are not known. The pH-dependence results, discussed below, suggest that salt bridges rather than non-H-bonded ion pairs are responsible for the helix stabilization. The reason for helix stabilization in the i+4 peptides relative to the i+3 peptides probably involves the ease of making H bonds between preferred rotamer conformations of glutamic and lysine side chains in the i+4 and i+3 peptides, respectively.

Even the i+3 peptides, which are weak helix-formers, show much more helix content than is predicted by the Zimm–Bragg equation. The helix content derived by their equation for a 16-residue alanine helix is ≈4%, with s = 1.1 and $\sigma = 8 \times 10^{-4}$ (taken from host–guest data) (11), whereas the helix contents of the i+3 peptides range from about 25% to 50% for K,E and E,K in 0.01 M NaCl. This suggests that there may be some helix stabilization provided by salt bridges or ion pairs in the i+3 peptides. These specific interactions are not taken into account in the Zimm–Bragg model and host–guest experiments. The pH titrations in Fig. 4 and the NaCl-dependence data in Table 1 do not, however, provide any direct evidence for salt bridges or ion pairs in the i+3 peptides. One might expect that if the i+3 peptides are stabilized by salt bridges in the same manner but to a lesser extent than the i+4 peptides, then the i+3 peptides should show similar (but reduced) pH and NaCl dependences. This is not the case. Peptides made by Bierzynski *et al.* (18) with the aim of testing whether a Glu^-···His^+ salt bridge (i+3 spacing) would stabilize an α-helix failed to show any helix formation. An alternative explanation for the unexpected stability of the i+3 peptides is that the helix-forming propensity of alanine peptides is greater than that predicted from the host–guest s and σ values for alanine in the Zimm–Bragg equation. Significant helix formation has also been observed for a different alanine-based peptide system in which a block of 20 alanine residues is solubilized by a block of 20 charged Glu^- residues (19). More work is needed to resolve this problem.

A statistical study (20) of Glu···Baa pairs in protein helices, where Baa is any basic residue (lysine, arginine, or histidine), gave an early indication that i+4 Glu^-···Lys^+ salt bridges are helix-stabilizing. When the spacing is i+4, Glu···Baa pairs occur with a statistically high frequency, but not when the spacing is i+3.

Remarkably, the difference in helix stability between the i+4 and i+3 peptides persists at pH 2 (where $Glu^- \rightarrow Glu^0$) and at pH 12 (where $Lys^+ \rightarrow Lys^0$). The simplest explanation is that the H bonds persist when one partner of the salt bridge is uncharged and that these singly charged H bonds (Glu^0···Lys^+ at pH 2, Glu^-···Lys^0 at pH 12) are roughly as effective as the Glu^-···Lys^+ salt bridge in stabilizing the helix. If this explanation is correct, then it suggests that salt bridges (H-bonded ion pairs) rather than nonbonded ion pairs are responsible for helix stabilization at neutral pH. Studies by site-directed mutagenesis of the H bonds involved in tyrosine activation by a tyrosyl-tRNA synthetase also indicate that singly charged H bonds are strong H bonds (21).

X-ray structural analysis has been used to investigate the role of salt bridges in protein structure and function [see the review by Perutz (22)]. One classic example is hemoglobin: the deoxy tetramer contains eight salt bridges not found in the oxy form, and these salt bridges are believed to be important in stabilizing the deoxy over the oxy form in the absence of O_2. Another well-known example is the internal salt bridge of α- or δ-chymotrypsin. Tryptic activation of chymotrypsinogen produces the new α-NH_3^+ group of Ile-16, which swings in to form a salt bridge with the β-COO^- group of Asp-194 (23). Formation of this salt bridge drives the conformational change responsible for activation of the proenzyme (24). The long, isolated, central helix of calmodulin or troponin C may be stabilized by intrahelical salt bridges (25). It has been possible to measure the strength of a protein salt bridge in two cases: ΔG° = −2.9 kcal/mol for the buried salt bridge of δ-chymotrypsin (26) and ΔG° = −1.0 kcal/mol for the more exposed salt bridge connecting the N and C termini of bovine pancreatic trypsin inhibitor (27).

pH Titration and NaCl Dependence of Helix Content. We expected that titration of the peptides to pH 2 or pH 12 would disrupt salt-bridge and ion-pair interactions and that (i+4)E,K and (i+3)E,K would show similar helix contents at these pH extremes, as would (i+4)K,E and (i+3)K,E. Instead we found that the peptides are almost as different in helicity at pH 2 or pH 12 as at pH 7, and we are forced to conclude that singly charged H bonds are comparable in importance to salt bridges. This surprising result is the main reason why it is difficult to interpret the pH-dependence curves, but there are also two other pH-dependent factors that affect helix stability: the change in s as a charged residue is neutralized (11, 28) and the change in helix dipole interactions as some of the charged groups are neutralized. These three factors can, moreover, have interdependent effects. Formation of a salt bridge pulls the charged side chains

together and away from the ends of the helix, which affects their interaction with the helix dipole. This effect probably explains why the helix dipole interaction seems to be smaller in the $i+4$ peptides than in the $i+3$ peptides.

The [NaCl]-dependence results are also not simple. The rationale for studying the dependence of helicity on [NaCl] is that charge–charge interactions typically can be screened by mobile counterions and the [NaCl] dependence tells whether the net interaction is attractive or repulsive. At first sight the results shown in Fig. 5*a* lend themselves to a simple explanation of this type. A bell-shaped curve with maximal helicity at pH 7 is found for $(i+4)$E,K at 0.01 M NaCl, whereas the curve is nearly flat at 1.0 M NaCl, suggesting that ion pairs or salt bridges stabilize the helix at pH 7 and that the attractive interaction can be screened almost completely by 1.0 M NaCl. But comparison of the $i+4$ and $i+3$ peptides indicates that ion pairs or salt bridges also stabilize the $(i+4)$K,E helix, and there is no significant change in its helicity at pH 7 between 0.01 M and 1.0 M NaCl (Table 1). Another confusing result is that the helicity of $(i+4)$E,K continues to change with [NaCl] > 1 M at pH 7.1 but is nearly independent of [NaCl] at pH 2.25 (Fig. 5*b*).

In summary, the data show that pH and [NaCl] are important variables affecting the helix contents of these peptides and that it would be very useful to understand these effects. At least three factors seem to be involved, and understanding their effects is not simple.

Enthalpy-Driven Formation of Peptide Helices. When formation of the C-peptide helix was found to be enthalpy-driven (4), it was possible to suppose that this was not general and that the cause lay in the specific side-chain interactions that stabilize this helix. In the case of the present alanine-based peptides, a different type of side-chain interaction ($Glu^{-}\cdots Lys^{+}$ salt bridges) is allowed. The most stable of these helices $[(i+4)$E,K] is characterized by the same type of thermal unfolding as the least stable $[(i+3)$K,E] (Fig. 3*a*). It seems probable, therefore, that some other factor, common to both sets of peptides, provides the enthalpic contribution to helix formation. The *s* values of the different amino acids do not indicate that helix formation should be strongly enthalpy-driven: many *s* values are only weakly dependent on temperature, and some increase with temperature (11).

There are two likely candidates for the enthalpic contribution to helix formation. One is a negative enthalpy change for peptide H-bond formation in H_2O, as suggested originally by Schellman (29), and a second likely candidate is the hydrophobic interaction, which is entropy-driven at room temperature (30) but which becomes increasingly enthalpy-driven at higher temperatures (31).

Concluding Remarks. These experiments show that it is feasible to study side-chain interactions involving charged residues in alanine-based peptides of *de novo* design. The results raise further questions, some of which should be answerable by the design and synthesis of new peptides. Do both $i+1$ and $i+4$ spacings contribute to the observed stabilization of the helix in the $i+4$ peptides? Can α-helix formation still be observed in alanine-based peptides in which intrahelical salt bridges and ion pairs are completely ruled out? Our results also raise other questions, which call for theoretical analysis. For instance, why do salt bridges in the $i+4$ peptides stabilize the helix more than in the $i+3$ peptides? Finally, these designed peptides should provide a model for testing electrostatic theories of charge–charge interactions in proteins.

We are grateful for the discussion of F. M. Hughson, P. S. Kim, I. D. Kuntz, C. R. Matthews, G. D. Rose, and A. B. Sachs and for experimental help from P. S. Kim. We thank Virginia MacCosham for technical assistance. This research was supported by National Science Foundation Grant DMB 85-18112. S.M. is a predoctoral fellow of the Medical Scientist Training Program of the National Institutes of Health (GM07365).

1. Schechter, B., Schechter, I., Ramachandran, J., Conway-Jacobs, A. & Sela, M. (1971) *Eur. J. Biochem.* **20,** 301–308.
2. Ramachandran, J., Berger, A. & Katchalski, E. (1971) *Biopolymers* **10,** 1829–1851.
3. Goren, H. J., Fridkin, M., Katchalski-Katzir, E. & Lotan, N. (1979) *Biopolymers* **18,** 981–993.
4. Bierzynski, A., Kim, P. S. & Baldwin, R. L. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2470–2474.
5. Shoemaker, K. R., Kim, P. S., Brems, D. N., Marqusee, S., York, E. J., Chaiken, I. M., Stewart, J. M. & Baldwin, R. L. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2349–2353.
6. Zimm, B. H. & Bragg, J. K. (1959) *J. Chem. Phys.* **31,** 526–535.
7. Brown, J. E. & Klee, W. A. (1971) *Biochemistry* **10,** 470–476.
8. Shoemaker, K. R., Kim, P. S., York, E. J., Stewart, J. M. & Baldwin, R. L. (1987) *Nature (London)* **326,** 563–567.
9. Rico, M., Gallego, E., Santoro, J., Bermejo, F. J., Nieto, J. L. & Herranz, J. (1984) *Biochem. Biophys. Res. Commun.* **123,** 757–763.
10. Scheraga, H. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 5585–5587.
11. Sueki, M., Lee, S., Powers, S. P., Denton, J. B., Konishi, Y. & Scheraga, H. A. (1984) *Macromolecules* **17,** 148–155.
12. Chou, P. Y. & Fasman, G. D. (1974) *Biochemistry* **13,** 211–221.
13. Vasquez, M., Pincus, M. R. & Scheraga, H. A. (1987) *Biopolymers* **26,** 351–371.
14. Stewart, J. M. & Young, J. D. (1984) *Solid Phase Peptide Synthesis* (Pierce Chemical, Rockford, IL).
15. Rosen, H. (1957) *Arch. Biochem. Biophys.* **67,** 10–15.
16. Chen, G. C. & Yang, J. T. (1977) *Anal. Lett.* **10,** 1195–1207.
17. Holzwarth, G. & Doty, P. (1965) *J. Am. Chem. Soc.* **87,** 218–228.
18. Bierzynski, A., Dadlez, M., Sobocinska, M. & Kupryszewski, G. (1986) *Biophys. Chem.* **25,** 127–134.
19. Ihara, S., Ooi, T. & Takahashi, S. (1982) *Biopolymers* **21,** 131–145.
20. Maxfield, F. R. & Scheraga, H. A. (1975) *Macromolecules* **8,** 491–493.
21. Fersht, A. R., Shi, J.-P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. Y. W. & Winter, G. (1985) *Nature (London)* **314,** 235–238.
22. Perutz, M. F. (1978) *Science* **201,** 1187–1191.
23. Sigler, P. B., Blow, D. M., Matthews, B. W. & Henderson, R. (1968) *J. Mol. Biol.* **35,** 143–164.
24. Oppenheimer, H. L., Labouesse, B. & Hess, G. P. (1966) *J. Biol. Chem.* **241,** 2720–2730.
25. Sundaralingam, M., Drendel, W. & Greaser, M. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 7944–7947.
26. Fersht, A. R. (1972) *J. Mol. Biol.* **64,** 497–509.
27. Brown, L. R., De Marco, A., Richarz, R., Wagner, G. & Wüthrich, K. (1978) *Eur. J. Biochem.* **88,** 87–95.
28. Maxfield, F. R., Alter, J. E., Taylor, G. T. & Scheraga, H. A. (1975) *Macromolecules* **8,** 479–491.
29. Schellman, J. A. (1955) *C. R. Trav. Lab. Carlsberg Ser. Chim.* **29,** 223–229.
30. Kauzmann, W. (1959) *Adv. Protein Chem.* **14,** 1–63.
31. Baldwin, R. L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 8069–8072.