

Unusually stable helix formation in short alanine-based peptides

(model α -helix/protein folding/helix-coil transition)

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ABSTRACT Short, 16-residue, alanine-based peptides show stable α -helix formation in H₂O. This result is surprising when contrasted with the classical view that regards the α -helix as a marginally stable structure in H₂O and considers short helices unstable. The alanine-based peptides are solubilized by insertion of three or more residues of a single charge type, lysine (+) or glutamic acid (–). The results cannot be explained by helix stabilization resulting from concentration-dependent association or by the interaction of charged residues with the helix dipole. Our results are not predicted by the parameters for alanine and lysine that have been determined by the “host-guest” method: these parameters predict that a 16-residue peptide should not show measurable α -helix formation. Analysis of the role of the hydrophobic interaction in α -helix formation [Richards, F. M. & Richmond, T. (1978) in *Molecular Interactions and Activity in Proteins*, Ciba Foundation Symposium 60, ed. Wolstenholme, G. E. (Excerpta Medica Amsterdam), pp. 23–25] does not show an unusually strong hydrophobic interaction in a helical block of alanine residues. The likely explanation for our results is, therefore, that individual alanine residues have a high helical potential. It is not yet known whether any other amino acids show this property, and the origin of this property is also unknown.

The α -helix is the most abundant element of secondary structure in proteins (1); yet the α -helix has been regarded in the past as only marginally stable in H₂O. Studies of α -helix formation by long polypeptides, together with the Zimm-Bragg model, predict (2) that short protein fragments as well as other peptides should not show measurable helix formation in H₂O. The Zimm-Bragg helix-coil transition theory relates the helix content of a polypeptide to three parameters: s , the intrinsic helix-forming propensity of an amino acid; σ , the constant for nucleating the helix; and n , the number of peptide units in the polypeptide. This model neglects sequence- and position-dependent side-chain interactions. Studies with polypeptides, termed “host-guest” experiments, determine the Zimm-Bragg parameters for an individual amino acid by incorporating it randomly into a helix-forming polymer such as poly(hydroxybutyl-L-glutamine). Although the determined values of s for the 18 different amino acids studied by the host-guest method (3) are significantly different, these values are all close to 1 (0.6–1.3 at 20°C), values indicative of marginal helical stability even for long polypeptides. Experimental studies give values for $\sigma \leq 10^{-3}$ (4). These parameters indicate that no peptide <20 residues in length should exhibit measurable helix formation in H₂O at any temperature if the Zimm-Bragg model applies (2).

Whereas most reports do not show observable helix content in short protein fragments and other peptides, there are now several examples in which helix formation has been detected. The C- and S-peptide fragments of ribonuclease A (residues 1–13 and 1–20, respectively) were the first examples

of this kind (5–8). At low temperature (3°C) and pH 5, the C peptide was found to have $\approx 25\%$ helix content (6). Recently we showed that simple peptide sequences of *de novo* design, containing chiefly alanine with inserted pairs of glutamic and lysine residues, can form quite stable α -helices (9). The inserted pairs of glutamic and lysine residues are spaced three or four amino acids apart to permit ion-pair formation, and helix stabilization by ion pairs is clearly observed with the “ $i+4$ ” spacing. In all examples to date of short peptide helices in H₂O, helix stabilization by sequence- or position-specific side-chain interactions, such as ion-pair and charged group-helix dipole interactions, is strongly implicated. It is not clear, however, whether all of the unexpected helical stability in these peptides can be attributed to specific side-chain interactions. Therefore, we ask what helix content these short peptide sequences would show in the absence of side-chain interactions.

In this study, our goal is to determine the helix-forming properties of individual residues in short peptides, beginning with alanine. We show here that helix formation is striking in peptides that contain only alanine plus a small number of residues of a single type of charged amino acid (lysine or glutamate). The resulting helices are monomolecular, and the design of the peptides is such that the results cannot be explained by ion-pair formation or by charged group-helix dipole interactions. Our results indicate that alanine itself can stabilize short helices. This finding clashes with the classical view that, because hydrogen bonds between the peptide group and H₂O compete favorably with helical peptide hydrogen bonds (10), the α -helix is intrinsically a marginally stable structure in H₂O. Moreover, the Zimm-Bragg parameters for alanine obtained by the host-guest method are small, $s = 1.07$ and $\sigma = 8 \times 10^{-4}$ (20°C) (11), and suggest that short alanine peptides should not show observable α -helix content in H₂O (2).

MATERIALS AND METHODS

Peptide Design. Because most helix-favoring amino acids are hydrophobic, special attention is needed in the design of peptides to ensure that they are H₂O-soluble. Alanine is found frequently in protein helices but oligo(L-alanine) peptides are not H₂O-soluble. Ooi and coworkers (12) have studied a single block of (Ala)₂₀ solubilized by an adjacent block of (Glu)₂₀. In our designed peptides, charged residues are inserted within a short alanine block to solubilize it. When this approach is used, it is important to avoid sequences that form amphiphilic helices and also sequences in which hydrophobic and hydrophilic residues alternate because these sequences tend to form β -sheets (13, 14). An amphiphilic helix tends to self-associate along the hydrophobic face of the helix. Thus, Ho and DeGrado (15) have designed peptides that form amphiphilic helices and self-associate to form four-helix bundles. The helix is stabilized by association,

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Abbreviation: TFE, trifluoroethanol.

3K (I)	Ac - A A A A K A A A A K A A A A K A - NH ₂
3K (II)	Ac - A K A A A A K A A A A K A A A A - NH ₂
4K	Ac - A K A A K A A A A K A A A A K A - NH ₂
6K (I)	Ac - A K A A K A K A A K A K A A K A - NH ₂
6K (II)	Ac - A K A A A K K A A A K K A A A K A - NH ₂
3E	Ac - A E A A A A E A A A A E A A A A - NH ₂
(i+3) E,K	Ac - A E A A K A E A A K A E A A K A - NH ₂
(i+4) E,K	Ac - A E A A A K E A A A K E A A A K A - NH ₂

FIG. 1. Sequences of the six alanine-based peptides compared with sequences of two peptides designed to allow ion pair formation. Peptides were also synthesized with tyrosine at position 1 for concentration determination (see *Materials and Methods*). Ac, acetyl; A, alanine; K, lysine; and E, glutamic acid.

which can be measured from the concentration dependence of helix stability.

The peptides studied here have sequences based on our previous design of helix-forming peptides that have the potential to form ion pairs (9). Fig. 1 illustrates the peptide sequences studied. The sequences of two of the original ion-pair peptides are also listed for comparison. To measure the helix-forming properties of an individual amino acid, it is necessary to avoid helix stabilization by ion pairs and other specific interactions between side chains. Thus, the solubilizing residues used here belong to a single charge type, either lysine or glutamic acid. The designed peptides contain 16 (or 17) residues—chiefly alanine—with 3 to 6 charged residues inserted. The charged residues spiral around the α -helix to avoid forming an amphiphilic helix and to help solubilize the otherwise insoluble block of alanine. Association or aggregation can be tested by studying the concentration dependence of the helix-coil transition, and the results are compared with data for four-helix bundles (15) and coiled-coil dimeric helices (16).

Charged groups can interact with a nearby pole of the helix dipole; this interaction will be helix-stabilizing when the two are of opposite sign and helix-destabilizing when they are of like sign. This phenomenon has been termed a "charged group-helix dipole" interaction. Two types of tests have been made here to determine whether charged group-helix dipole interactions are solely responsible for helix formation in these peptides. (i) Two different peptides have been designed with three solubilizing lysine residues, 3K(I) and 3K(II). The peptide 3K(II) contains a lysine near the N terminus or positive pole of the helix dipole, whereas 3K(I)

contains a lysine near the C terminus or negative pole. (ii) Charged group-helix dipole interactions are also evaluated by examining the pH dependence of helix formation.

Peptide Synthesis. All peptides except for 3K(II) were synthesized by solid-phase peptide synthesis on a DuPont 2100 coupler with conventional *N*-*tert*-butyloxycarbonyl (*t*-Boc) chemistry (17). Peptide 3K(II) was synthesized on a Milligen 9050 synthesizer using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. Couplings were monitored by the Kaiser test (17), repeated if necessary, and finally capped with acetic anhydride. Peptides were synthesized as C-terminal amides on *p*-methylbenzhydrylamine (polystyrene/1% divinylbenzene) resin. To determine peptide concentrations quantitatively, all peptides were synthesized with a tyrosine at position 1 and, for comparison, a second set was made with alanine at position 1. Because solid-phase peptide synthesis proceeds from the C terminus toward the N terminus, these two sets of peptides are easily synthesized by simply removing half the resin before the final N-terminal coupling cycle. Peptides synthesized by the *t*-Boc method were cleaved from the resin using a modified "low-high" trifluoromethanesulfonic acid-cleavage protocol (18).

Peptide Purification. Peptides were purified first by gel filtration chromatography on a Sephadex G-25sf column in 10 mM ammonium acetate, pH 4.75, and then by reverse-phase chromatography on C₁₈ resin by using a gradient of 0–40% acetonitrile in 0.1% trifluoroacetic acid. Peptide purity and composition were determined by fast protein liquid chromatography (Pharmacia) and amino acid analysis. The primary-ion molecular weight was determined by fast-atom bombardment mass spectrometry.

Circular Dichroism (CD) Measurements. CD spectra were taken on an Aviv 60DS spectropolarimeter. Samples were prepared as described (9). To determine peptide stock concentration accurately, the set of peptides with tyrosine at position 1 was used. Stock peptide concentration was then determined by measuring tyrosine absorbance in 6 M guanidine hydrochloride at 275 nm ($\epsilon_{275} = 1450$) (19). CD measurements on the set of peptides containing alanine at position 1, such as pH dependence of helix formation, are then scaled to match the signal obtained with the corresponding peptide containing tyrosine 1. The assumption that the two peptides will show the same CD intensity at wavelengths where the tyrosine side chain does not contribute to the spectrum and at pH values where the tyrosine is not ionized is based on the finding (20) that in the C-peptide helix residue 1 is frayed and makes little contribution to helix stability.

In our previous studies, peptide concentration was determined by ninhydrin analysis of hydrolyzed peptide samples. Measuring the tyrosine absorbance was found to be both

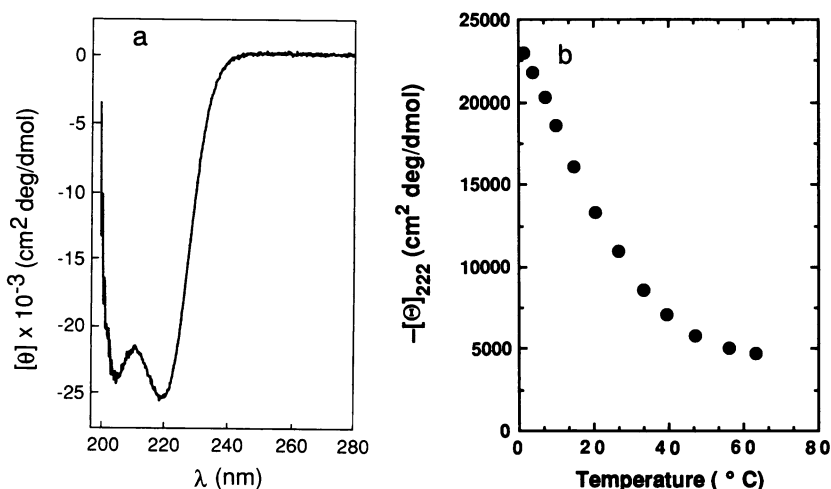


FIG. 2. (a) CD spectrum of the peptide 3K(I) (20 μ M) in 1.0 M NaCl (pH 7.0, 1.0°C). (b) Thermal unfolding of the peptide 3K(I) as measured by $-[\theta]_{222}$, the mean residue ellipticity at 222 nm (0.01 M NaCl, pH 7.0).

Table 1. Helix content at pH 7 and 1.0°C

Peptide	$-\theta_{222}$ (cm ² -deg-dmol ⁻¹)	
	0.01 M NaCl	1.0 M NaCl
3K(I)	22,900	25,100
3K(II)	21,300	24,800
4K	17,300	22,700
6K(I)	6,100	13,600
6K(II)	8,300	18,000
3E	22,600	21,000

more accurate and more precise than the ninhydrin method. Results obtained by ninhydrin analysis were on average 10–20% lower than those obtained by tyrosine absorbance (this corresponds approximately to 10–20% higher values of mean residue ellipticity). Therefore, values from our previous studies are probably too high and are less accurate than those given here.

Trifluoroethanol (TFE = CF₃CH₂OH) was 99+% pure from Aldrich. pH determination in samples containing TFE was made as described by Nelson and Kallenbach (21).

RESULTS

Helix Formation. Peptide helix formation is monitored by CD. At low temperature (1°C) all peptides show spectra indicative of an α -helix; they have the characteristic double minima at 222 nm and 208 nm (Fig. 2a). The extent of helix formation is most easily monitored by following the minimum at 222 nm, $-\theta_{222}$. Table 1 lists helix content measured by $-\theta_{222}$ in both 1.0 and 0.01 M NaCl (1°C, pH 7) for the peptides studied. All peptides show significant helix formation; $-\theta_{222}$ varies from 6,100 to 25,100 cm²-deg-dmol⁻¹, indicating \approx 15–80% helix content (see estimates below based on extrapolation from peptides in high TFE concentrations).

Helix formation is an enthalpy-driven process. Unfolding increases with temperature in the same manner observed

previously for designed peptides with ion pairs (9) and for the C peptide and its analogs (5–8) (Fig. 2b). The thermal unfolding transition is very broad, spanning $>70^\circ\text{C}$. The absence of a plateau at low temperatures is consistent with the observation that helix formation is only partial under these conditions.

Concentration Dependence of Helix Formation. Studies of helix formation as a function of peptide concentration indicate that the helix-forming unit is monomeric. Fig. 3a shows the lack of concentration dependence of $-\theta_{222}$ for peptide 3K(I) in helix-forming conditions, 1°C and pH 7, as expected for a monomeric species. Fig. 3b illustrates a similar test for concentration-dependence made throughout the unfolding transition zone, using guanidine hydrochloride as a denaturant, to ensure that the test is made at the level of stability most sensitive to changes in peptide concentration. The CD signal is independent of peptide concentration at all denaturant concentrations. This approach is illustrated in Fig. 3c and d with results from the literature for a system in which helical stability does depend on concentration. The peptide associates into four-helix bundles (15), and helix formation is strongly dependent on concentration throughout the whole range of helix stability. In dimeric α -helices that form coiled coils, no dimer helix formation has been seen with peptides shorter than 29 residues (16).

Effect of the Number of Charged Residues. The role of the lysines in affecting helix formation was examined by varying their number (three, four, or six) and position within the sequence (Table 1). At neutral pH, the best helix formers are 3K(I) and 3K(II); $-\theta_{222} = 25,100$ and $24,800$ cm²-deg-dmol⁻¹, respectively, at 1°C, 1.0 M NaCl. Adding another lysine, as in peptide 4K, results in a small decrease in helix stability. Peptides containing six lysines show, however, a substantial decrease in helix content.

Effect of the Type of Charged Residue (Lysine and Glutamic Acid). The specific effect of the charged residue was evaluated by changing the nature of the charged group from positively charged lysine to negatively charged glutamate. At neutral pH a peptide with three glutamic residues shows a

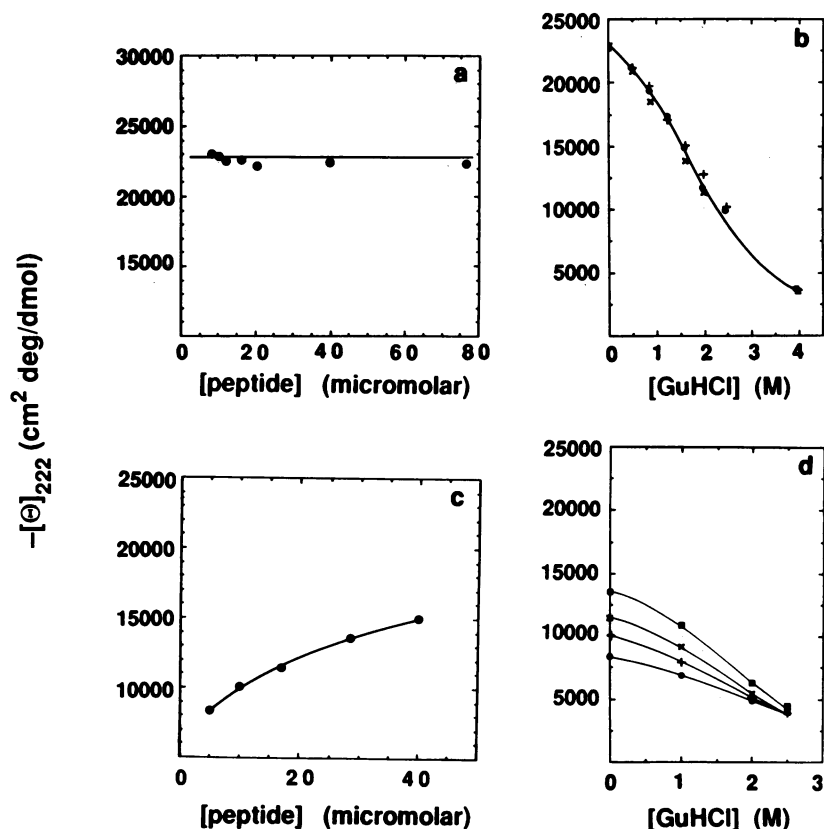


FIG. 3. Test for dependence of helix formation on peptide concentration for the peptide 3K(I) (a and b) and for the peptide α_1A designed by DeGrado and coworkers (15) to associate into a four-helix bundle (c and d). These figures illustrate by comparison that helix formation in the alanine-based peptide is monomolecular; data for c and d were taken from ref. 15. (a) Dependence of helicity on peptide concentration for the peptide 3K(I) (0.01 M NaCl, pH 7, 1°C). (b) Dependence of helicity on concentration of guanidine hydrochloride at 16.3 μM (\bullet), 8.2 μM (+), and 4.1 μM (\times) 3K(I) at pH 7 and 1°C. (c) Dependence of helicity on peptide concentration for the peptide α_1A . (d) Dependence of helicity on concentration of guanidine hydrochloride at several concentrations of α_1A : 5 μM (\bullet), 10 μM (+), 17 μM (\times), and 28.5 μM (\blacksquare).

similar helix content as either peptide with three lysines. Thus, peptides with either three glutamic or three lysine residues show unusually stable helix formation.

pH Dependence of Helix Formation. The importance of charge effects on helix content can be evaluated by measuring helix formation as a function of side-chain ionization or pH. Fig. 4 shows the pH dependence of $-\theta_{222}$ at 1°C and 0.01 M NaCl for three lysine-containing peptides. All three peptides show qualitatively similar curves independent of the specific placement of the lysine residues. The helix is least stable at neutral pH, and helicity increases in the basic range. The apparent pK indicates that this effect arises from titration of the ϵ -amino group of lysine. Peptide association at high pH (>10) has not been studied.

Effect of TFE. TFE, an organic solvent known to promote helix formation in peptides (21), was used to obtain the value of $-\theta_{222}$ for maximal helix formation by each peptide. Helix content as a function of mol % TFE is shown in Fig. 5 for three peptides containing three, four, or six lysines. At high concentrations of TFE, all three peptides display the same CD spectrum and $-\theta_{222}$ reaches the same maximum value of $\approx 32,000 \text{ deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$. This value is reasonable for 100% helix formation in peptides of this length. Moreover, because all peptides show the same value of $-\theta_{222}$ at sufficiently high TFE concentrations, the different values of $-\theta_{222}$ (and also the different spectral features) seen at 0% TFE must reflect true differences in helicity and not just differences resulting from sequence-dependent spectral properties.

DISCUSSION

Unusual Peptide Helix Stability. These peptides form surprisingly stable α -helices. Under optimal helix-forming conditions, 16-residue peptides containing 3 lysines and 13 alanines show as much as 80% helix. Such stable helix formation in a short peptide has been seen previously only in designed peptides stabilized by salt bridges between glutamic acid and lysine four residues apart ($i+4$). Although these new peptides are compositionally quite similar to those, most of the stable peptides studied here contain a higher content of alanine. Among these peptides, helix stability parallels the alanine content. Peptides with three lysines form more stable helices than those with four or six lysines. Comparison of peptides with three glutamic residues (3E) to those with three lysines [3K(I) and 3K(II)] shows little effect of substituting charged glutamate for charged lysine. We conclude that a dominant factor in these peptides is the high alanine content and that alanine is a strongly helix-favoring amino acid. It is

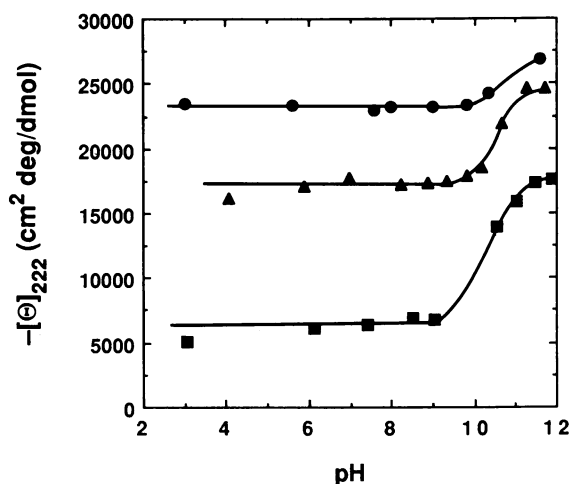


FIG. 4. pH titration of helicity as measured by $-\theta_{222}$ for three lysine-containing peptides—3K(I) (●), 4K (▲), and 6K(I) (■)—in 0.01 M NaCl at 1°C.

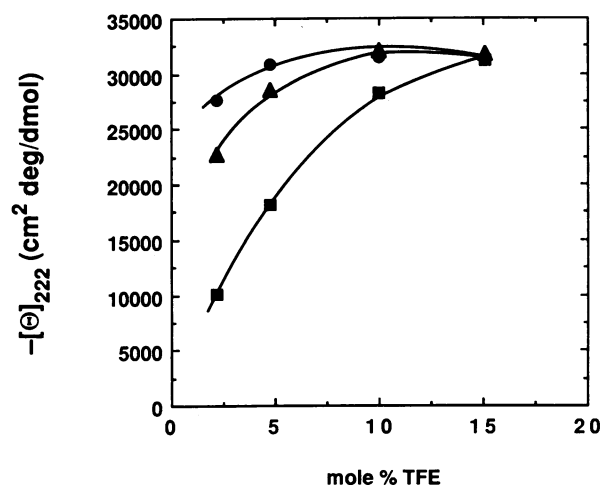


FIG. 5. Dependence of helicity on TFE concentration for three lysine-containing peptides—3K(I) (●), 4K (▲), and 6K(I) (■)—at pH 7 and 1°C.

no longer surprising, therefore, that in our previous work (9) peptides with glutamic and lysine pairs spaced three amino acids apart ($i+3$; Fig. 1) form moderately stable helices (25–50%) even though in the ($i+3$) spacing there is no evidence of stabilization by salt bridges.

Possible Explanations of the Unusual Helix Stability. The most likely explanation for our results is that individual alanine residues have a high helix-forming potential. There are three obvious types of side-chain interactions, however, that might provide an alternative explanation to these results, but each of these interactions can be eliminated. (i) Lateral association between helices is known to be helix-stabilizing when it occurs (15, 16). This interaction can be ruled out because we find convincing evidence that helix formation is monomolecular (see Fig. 3 and *Results*). (ii) Charged-group-helix dipole interactions are known to stabilize short helices (22, 23). This interaction cannot be the sole explanation for stable helix formation for the following reasons. Peptides 3K(I) and 3K(II) contain a positively charged group at opposite ends of the helix, yet both peptides show substantial helix formation. Moreover, helix stability increases as the charge is removed from the lysine residues by pH titration (Fig. 4), whereas charged group-helix dipole interactions must disappear as the charge disappears. Note that helix stabilization by ion pairs also can be ruled out because these peptides contain charged residues of only one charge type. (iii) Hydrophobic interactions can result from partial burial of nonpolar surfaces when helix formation occurs. This factor has been discussed often, but experimentally separating it from other factors affecting helix stability is difficult. This subject is considered in a separate section (see below).

Comparison with Earlier Work. Unusual helical stability for a block of L-alanine residues has been observed in earlier studies (24, 25) beginning with the pioneering study by Gratzer and Doty (24) in 1963. Blocks of D,L-glutamate (24) or D,L-lysine (25) were used to solubilize the alanine blocks.

Two explanations have been offered for the perplexing stability of the alanine helix in these block copolymers. In the longer block copolymers, self-association of alanine blocks within one polymer molecule may occur (25). In shorter block copolymers, where lateral association between alanine helices within one polymer is unlikely, a helix-stabilizing hydrophobic interaction has been postulated (25, 26) (see section below).

Later Ooi and coworkers (12) studied helix formation by a single block of 20 alanine residues solubilized by attachment to an adjacent block of 20 ionized L-glutamic residues. The primary aim was to find out whether the helical stability of the

(Ala)₂₀ block depends on whether the (Glu)₂₀ block is attached at the N-terminal or C-terminal end of (Ala)₂₀. A strong position effect was found (12). Their study also showed that the alanine block has a high helical stability. The alanine block is estimated to show ≈90% helix formation if one assumes that only the alanine block is helical at neutral pH. Although a (Glu)₂₀ block would not form an α -helix by itself when ionized, the helix, once formed, could propagate from the alanine block into the (Glu)₂₀ block. Therefore, this unusual stability was not considered surprising because the peptides contained only L-amino acids and were 41 residues long, which strongly increases the helical probability.

Role of the Hydrophobic Interaction in Stabilizing Isolated α -Helices. The notion that the hydrophobic interaction may be an important determinant of α -helix stability has a long history [see the 1972 discussions by Fasman and coworkers (27) and by Ptitsyn (4) of their own and earlier work]. It became possible to discuss quantitatively the role of the hydrophobic interaction in protein folding reactions when Lee and Richards (28) developed an accurate algorithm for computing H₂O-accessible surface area and Chothia (29) correlated transfer free energies with nonpolar accessible surface area for amino acid side chains. Then, assuming that the same correlation applies to folding reactions, one can compute the free energy change corresponding to the burial of hydrophobic groups upon folding.

Richards and Richmond (30) give data for this decrease in nonpolar accessible surface area when a given amino acid residue folds into an α -helix, in which the surrounding residues are either alanine or the sequences found in the helices of sperm whale myoglobin. They find that a decrease in nonpolar accessible surface area always accompanies α -helix formation, that the typical change in Gibbs free energy is modest [≤ 0.5 kcal per residue (1 Cal = 4.184J) for amino acids with small side chains], and that the change does not vary strongly from one residue to the next within a given size class. The change found for alanine is not exceptional. Moreover, the difference in the value obtained using either an alanine helix as background or the average of the myoglobin helices is small. The explanation given earlier (26) of the unusual helical stability noted in polymers containing blocks of alanine is based on a specific hydrophobic contact between the α carbon of residue i and the β carbon of residue $i+3$. Such an interaction would not be specific for alanine but would be allowed for all residues containing a β carbon and should also occur between alanine and the host residue (hydroxypropyl-L-glutamine) used in the host-guest study of alanine (11) (see the following section).

Consequently, the probable conclusion from our study is that individual alanine residues have a high intrinsic helical potential. Whether alanine is exceptional in this respect or whether other nonpolar residues also have high helical potentials remains to be determined.

Comparison with Host-Guest Results. The value of s for alanine given by the host-guest method is 1.08 at 0°C (11), whereas a preliminary analysis of our results by the Lifson-Roig theory (31), in collaboration with J. A. Schellman, gives s (0°C) ≈ 2, or a 2-fold difference. (This preliminary analysis uses the host-guest value of s for lysine, which is 0.94 at 20°C.) In 1966 Berger and coworkers (10) studied the helix-forming properties of HBLG (hydroxybutyl-L-glutamine) and HPLG (hydroxypropyl-L-glutamine), the host residues of host-guest studies, and HELG (hydroxyethyl-L-glutamine). They found that HBLG forms a moderately stable helix in H₂O, but that HELG, like poly(L-glutamine) itself, does not form a helix in H₂O. HPLG forms a helix of intermediate stability. All three derivatives—HBLG, HPLG, and HELG—show stable helix formation in organic solvents such as methanol. Berger and coworkers (10) concluded that the HBLG helix is stabilized by hydrophobic interactions be-

tween adjacent hydroxybutyl side chains. Accepting their explanation, we suggest that in the copolymer of alanine and HPLG used in host-guest studies of alanine (11), the short alanine side chain is unable to take part in the hydrophobic interaction that involves the hydroxypropyl moiety of the HPLG residue. This failure to participate in a helix-stabilizing interaction among host residues thus appears as a helix-destabilizing factor in the helical potential of alanine found by the host-guest method. The result is that the helical potential of alanine is underestimated.

It is important now to test this and other possible explanations for the difference between our results and those of Scheraga and coworkers (11); they note internal consistency between their host-guest values and the earlier block copolymer results by Ingwall *et al.* (25). The exact basis for this contradictory behavior is important for understanding the α -helix and remains a goal for the future.

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