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Reflections

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In Search of the Energetic Role of Peptide Hydrogen Bonds

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Robert L. Baldwin

From the Department of Biochemistry, Beckman Center, Stanford School of Medicine, Stanford, California 94305

Forming peptide hydrogen bonds was considered to be probably the most important driving force for protein folding in 1951, when Linus Pauling and Robert Corey proposed the hydrogen-bonded structures of the α-helix (1) and two β-sheets (2). Because the peptide CO and NH groups form competing hydrogen bonds (H-bonds) to water when a protein is unfolded it was evident, however, that the net contribution of the peptide H-bond (CO···HN) to protein stability might be small. In 1955 John Schellman (3, 4) made a first analysis of the energetics of peptide H-bonds in protein folding reactions. He listed the factors that should affect the stability of a peptide α-helix in water, and he estimated the strength of the peptide H-bond in water by attributing the unusual thermodynamics of aqueous urea solutions (which had been measured accurately) to H-bonded urea dimers. His analysis indicated that a peptide helix in water should have at most marginal stability. Any observable helix formation should be driven by the net enthalpy change (ΔH) of forming the peptide H-bond in water, and the aqueous urea data gave $\Delta H = -1.5$ kcal/mol. The proposal of Schellman that helix formation should be driven by the enthalpy of the peptide H-bond was adopted by Zimm and Bragg (5) and by Lifson and Roig (6) in their treatments of the statistical mechanics of helix formation. Klotz and Franzen (7) found, however, that the dimerization of *N*-methylacetamide (NMA) in water was too small to measure, and the energetic significance of peptide H-bonds for protein folding lapsed into uncertainty. Interest in the problem diminished further as support grew for the bold proposal by Walter Kauzmann (8) in 1959 that the hydrophobic interaction provides the major driving force for protein folding.

Hydrogen Bond Inventory

The change in internal energy (ΔE) for forming a hydrogen bond in the gas phase can be calculated by quantum mechanics, and with steady improvement in methods of calculation, calculated values of H-bond energies are now believed to be comparable in accuracy to good experimental values. A recent calculated value for the ΔE of dimerization of NMA in vacuum is -6.6 kcal/mol, which has been used as a model for the peptide H-bond (9). Some older calculated values indicate that the H-bonds formed by amide CO and NH groups to water (W), and also the W···W and CO···NH H-bonds, have roughly equal energies of about -6 ± 1 kcal/mol (10, 11). The conclusion a decade ago was that *a hydrogen bond inventory,* discussed by Alan Fersht (12, 13), is sufficient to describe the net enthalpy of the peptide H-bond in water. In writing the inventory, the number of H-bonds is assumed to be the same, whereas their types are similar on both sides of the equation.

$$\text{CO···W} + \text{NH···W} = \text{CO···HN} + \text{W···W} \qquad \text{(Eq. 1)}$$

If the assumptions made in writing Equation 1 are valid, then the net enthalpy of the peptide H-bond in water is 0 ± 1 kcal/mol to a first approximation. In making quantum mechanics calculations to model the peptide H-bond and the NH···W and CO···W H-bonds, the energies

found by using different model compounds to estimate one type of H-bond differ by as much as the energies found for different types of H-bond (11). The enthalpy change ΔH and the internal energy change ΔE are considered here to be interchangeable in aqueous solution because any difference between them should be negligible.

Note, however, that even a small net enthalpy change per peptide H-bond could be important because a typical protein forms about 70% of the possible peptide H-bonds. The patterns of peptide H-bonds found in proteins are complex (14), and any average number must be considered as a rough estimate. If ΔH is as large as -1 kcal/mol per H-bond, this projection would contribute -70 kcal/mol to the folding reaction of a protein with 100 peptide groups. The net free energy change ΔG for folding is in the range -5 to -10 kcal/mol for typical small proteins, and a -70 kcal/mol contribution would be highly significant. For comparison, the hydrophobic interaction is estimated to contribute about -126 kcal/mol to the folding reaction of a protein with 100 peptide bonds and 101 residues. The estimate of -1.2_5 kcal/mol residue is based on the average amount of nonpolar surface area buried per residue in the folding reaction of a typical protein ($\sim$50 Å^2 (15, 16)) and on a conversion factor between free energy and buried nonpolar area of 25 cal/Å^2. If the energy of the H-bond is -6 kcal/mol, it would be highly unfavorable to bury a peptide NH or CO group in the interior of a protein without making its H-bond, and burial of a free NH or CO group rarely occurs (14).

Use of the Alanine Peptide Helix to Analyze H-bond Energetics

A new approach to understanding peptide H-bond energetics became possible with the discovery by Susan Marqusee (17) that the alanine peptide helix is stable by itself in water. Curiously only alanine, of the 20 naturally occurring amino acids, has this property (18). Thus, helices formed by alanine-based peptides can provide absolute helix propensities when the effect of a guest residue is studied, whereas other systems typically give only relative values because the helix must be stabilized by some other interaction(s), such as salt bridges, whose strength is difficult to measure independently. Amino acids with larger nonpolar side chains, such as leucine, form less stable helices than alanine (18), which suggests that alanine helix formation is not driven by the hydrophobic interaction. Because alanine has just a methyl side chain and only a small amount of nonpolar surface area is buried when an alanine peptide forms a helix (19), it is unlikely at first sight that the hydrophobic interaction could be responsible for helix formation. Instead, the peptide H-bond and solvation of the peptide group probably drive alanine helix formation. Recent calorimetric studies (see below) demonstrate that in fact the hydrophobic interaction is not responsible for alanine helix formation. Alanine helices are prone to be water-insoluble, but two charged ornithine residues at either end of a 13-residue alanine sequence are sufficient to solubilize the helix (20).

The stability of a peptide α-helix formed from a single type of amino acid depends on two different equilibrium constants. The first is a nucleation constant for a reaction in which three adjacent peptide groups assume a helical conformation but no peptide H-bonds are formed, and the second is a helix propagation constant (the helix propensity) for a reaction in which a single H-bonded helical residue is added (5). A peptide H-bond is formed between the CO of peptide group i and the NH of peptide group $i + 3$. To find the absolute value of the helix propensity from measurements of helix stability, it is necessary to determine the helix nucleation constant, which is found from measurements of helix stability for a set of peptides of varying chain lengths. The ratio of helical residues formed in the nucleation reaction to those formed in the propagation reaction decreases as the helix becomes longer and more H-bonded residues are added, and so overall helix stability increases with chain length. The nucleation constant for an alanine-based helix was initially determined by Marty Scholtz (21), who used circular dichroism to measure the thermal unfolding curves of a set of peptides of chain lengths varying from 14, 20, 26 . . . to 50 residues. Later, Carol Rohl (22) confirmed the value of the nucleation constant by a new method, based on NMR measurement of the kinetics of hydrogen exchange, using a different peptide series (22). When we analyzed these results by an adaptation (23) of the Lifson-Roig theory (6), which is particularly well suited to analyzing helix formation by peptides with mixed sequences (24), the helix nucleation constant was found to be 0.0013, more than 1000-fold lower than the helix propagation constant for alanine, which is 1.70 at 0 °C (18).

The Enthalpy of Helix Formation

In using the alanine peptide helix as a wedge to pry open the energetics of peptide H-bonds, the first problem was to determine accurately the net enthalpy change for helix formation. Thermal unfolding curves (21) show that alanine helices unfold with increasing temperature, which suggests that that helix formation is enthalpy-driven. A standard method for measuring the enthalpy change on protein unfolding is differential scanning calorimetry (16). Thermal unfolding curves of peptide helices are quite broad, however, and the calorimetrically measured unfolding curve of even a 50-residue alanine-based helix (25) fails to show either 100% helix at 0 °C or 0% helix at 80 °C, the highest temperature reached in this experiment. The broad thermal melting curve prevents fitting the base line reliably, which limits the accuracy of measuring the enthalpy change. Nevertheless, we were able to measure, in collaboration with Wayne Bolen, an approximate value of the enthalpy change, −1.0 kcal/mol residue (25), which agrees with the value found by fitting helix melting curves (21). Unfortunately the broad melting curve is unsuitable for measuring ΔCp, the change in heat capacity on unfolding, which is the critical quantity for determining if the hydrophobic interaction drives folding. If helix formation is driven by the hydrophobic interaction, then a large ΔCp dominates the expression describing the thermal unfolding curve.

Recently, a way around the problem of the broad curves for thermal unfolding was found. In a peptide system developed by Andrzej Bierzynski and his co-workers (26), based on a peptide sequence taken from an EF-hand protein, helix formation occurs on adding La^{3+} so that ΔH can be measured by titration calorimetry. Independent measurements by us, in collaboration with George Makhatadze (27), gave $\Delta H = -0.90 \pm 0.1$ kcal/mol residue, in good agreement with the value of -0.98 ± 0.1 kcal/mol residue measured by Bierzynski and co-workers.[1] Moreover, measurements of ΔH made at two temperatures show that ΔCp is zero within error (27), which confirms that folding is not driven by the hydrophobic interaction. In the following, an average value of ΔH from these two studies is used, $-0.9_5 \pm 0.1$ kcal/mol residue. Using this value as the ΔH for the peptide H-bond in protein folding gives the scenario mentioned above, in which (even though ΔH per H-bond has only a modest value) the large number of peptide H-bonds (~70 for a protein with 100 peptide bonds) should produce a large enthalpic contribution, −66.5 kcal/mol, to the folding reaction if the assumptions of the H-bond inventory are applicable.

Application of H-bond Inventory to the Alanine Helix and to Amide Solvation

Does the H-bond inventory approach (Equation 1) give a correct prediction of the measured value of ΔH for alanine helix formation, as it should if peptide H-bonds and solvation of the peptide group provide the only important contributions to ΔH? First an important question must be answered: does the free peptide group form one or two H-bonds to water? As written above, Equation 1 implies that the peptide group makes only one H-bond to water, but both the peptide NH and CO groups should be able to form H-bonds to water. According to the H-bond inventory, the answer can be found from the enthalpy of solvation of simple amides, which must first be corrected for the contributions to the solvation enthalpy from van der Waals interactions and from making a cavity in water for the solute. (It is also necessary to use the correct standard state for transfer of a solute from the gas phase to liquid solution.) Calorimetric data for the solvation enthalpy of some amides are available when the starting material is the amide in gaseous form, and Peizhi Luo (29) analyzed the results to give the enthalpy of interaction between water and the amide polar groups. For four different amides, ΔH is close to −12 kcal/mol; the value for *N*-methylacetamide is −11.65 kcal/mol (29). Thus, following the rule discussed above in which ΔH for making one H-bond of this type is -6 ± 1 kcal/mol, we might at first conclude that the free peptide group makes two H-bonds to water (however, see below).

In any case, if we use the solvation enthalpy found with amides to predict the enthalpy of interaction between the free peptide group and water, then a simple enthalpy balance combined with the H-bond inventory gives ΔH for forming the alanine peptide helix as follows: ΔH(pred) = 12 (breaking H-bond(s) to water) − 6.6 (forming the peptide H-bond) − 6 (forming a W···W H-bond) = -0.6 ± 1, which agrees satisfactorily with the observed ΔH value for the alanine helix of $-0.9_5 \pm 0.1$ kcal/mol residue.

[1] G. Goch, M. Maciejczyk, M. Oleszczuk, D. Stachowiak, J. Malicka, and A. Bierzynski, submitted for publication.

However, when we use the H-bond inventory approach to predict the enthalpy of solvation of amides, we encounter a paradox, which I have discussed elsewhere (30). The solvation reaction, starting with the unsolvated amide (Am) in the gas phase (g), can be written as follows.

$$\text{HN-Am-CO (g)} + \text{W···W (l)} = \text{W···HN-Am-CO···W (l)} \quad \text{(Eq. 2)}$$

According to the H-bond inventory, the unsolvated amide breaks one W···W H-bond as it dissolves in liquid (l) water. Thus, the predicted enthalpy of solvating the two amide polar groups is −12 (making two H-bonds to water) + 6 (breaking one W···W H-bond) = −6 kcal/mol, but the observed value is −12 kcal/mol. If the assumptions of the H-bond inventory are used to argue that the dry amide makes only one H-bond when it dissolves in water, then the contradiction with the observed value of −12 kcal/mol is even worse. The predicted value then is −6 (making one H-bond to water) + 6 (breaking one W···W H-bond) = 0 kcal/mol. Because the H-bond inventory approach fails when applied to this simple problem, its validity is doubtful. Probably the main reason for its failure is that the role of water is not as simple as written in Equations 1 and 2, in which one W···W H-bond is broken per peptide H-bond when a protein unfolds or when a dry amide molecule is solvated (see the critique of the H-bond inventory by Ben-Naim (31)). A different approach to understanding the solvation enthalpies of amides is discussed next.

Electrostatic Approach to Understanding Amide Solvation

The standard approach used by chemists to understand the solvation of polar groups in model compounds is based on electrostatics (32, 33). The properties of the Born equation (1920) (34) provide a background for explaining why electrostatics are all important. Atom-splitting experiments were still a novelty in 1920, and Max Born was interested in why Wilson's cloud chamber can be used to see the tracks of charged particles in supersaturated water vapor. The path of a charged particle is visualized by the trail of water droplets it leaves behind. Born calculated the free energy ΔG of transferring an ion (with radius r, charge q) from vacuum to a continuum solvent (water) that has a dielectric constant D.

$$\Delta G = -(q^2/2r)[1 - (1/D)] \quad \text{(Eq. 3)}$$

He found that the free energy change enormously favors transfer of the ion from vacuum to water, regardless of whether the ion has a positive or negative charge, and consequently the ion induces the formation of water droplets. Chemists soon began to use Born's equation to analyze the solvation thermodynamics of ions in water. The enthalpy of electrostatic solvation can be predicted from Born's analysis and, in a solvent with a high dielectric constant such as water, the electrostatic solvation enthalpy of an ion is predicted to be almost equal to its electrostatic solvation free energy (35, 36). For amides, experimental values are available for both the enthalpy and free energy of solvating the amide polar groups, and the two quantities are nearly equal, probably within error (29). Consequently, the electrostatic solvation of the peptide CO and NH groups may be considered to be enthalpic when the solvation is correctly given by the electrostatic solvation free energy (ESF).

The solvation enthalpy of NaCl (infinite dilution) has the large value of −184 kcal/mol (35)! It is not surprising then that polar molecules such as amides, which contain large partial charges on the amide oxygen and nitrogen, have substantial solvation enthalpies associated with their partial charges, nor is it surprising that chemists have devised schemes for using electrostatics to compute the solvation free energies associated with polar groups. The calculation scheme devised by Barry Honig and his co-workers (32) has the attractive feature that the partial charges and atomic radii of the PARSE parameter set are calibrated from data for the solvation free energies of a large base of model compounds, including amides. The electrostatic algorithm DelPhi is used to compute the ESF. As discussed above, ESF values for the solvation of amide polar groups agree within error with the corresponding solvation enthalpies so long as the solvent is water.

Electrostatic Approach to Analyzing the Role of Solvation in Forming the Alanine Helix

The use of experimental solvation free energies to calibrate the parameters employed in calculating ESF values guarantees that the calculated ESF values will fit the experimental solvation free energies when applied to polar groups of the same type used for calibration. Will

electrostatic calculations also give meaningful ESF values when applied to related but different polar groups such as the peptide NH and CO groups in an alanine helix? The only groups that have significant partial charges in an alanine peptide with blocked end groups are the polar CO and NH groups. The ESF value of the central peptide group in a solvent-exposed, 15-residue alanine helix was computed by Franc Avbelj in collaboration with us (29) to be −2.5 kcal/mol, most of which (−2.0 kcal/mol) results from the interaction with water of the peptide CO group. Avbelj and I later found a similar result for peptide H-bonds in a β-structure, an alanine β-hairpin (37), namely that the H-bonded peptide group is solvated and its ESF value is about −2.5 kcal/mol. This calculated interaction between the H-bonded peptide group and water is a basic contradiction of the H-bond inventory.

Solvation of the peptide H-bond should have major energetic consequences for steps in the process of protein folding (i). There is a desolvation penalty for burying an H-bonded peptide group because the ESF value drops to zero when the peptide H-bond is buried out of contact with water (37). Thus, for an alanine helix, the desolvation penalty is exactly the opposite of the ESF of the peptide group in a solvent-exposed helix or 2.5 kcal/mol. The peptide H-bond in an alanine helix changes from being favorable for folding ($\Delta H = -0.9_5$ kcal/mol residue) when the H-bond is solvent-exposed to being unfavorable ($\Delta H = 1.5_5$ kcal/mol residue) when the helix is buried. Consequently, solvent-exposed peptide H-bonds should stabilize but buried H-bonds should destabilize (ii). The size of the calculated desolvation penalty (2.5 kcal/mol) is remarkably large compared with the numerically smaller free energy change per residue for burying the nonpolar surface during folding (−1.4 kcal/mol for an average residue). Consequently, the desolvation penalty ought to be a major factor limiting domain size in globular proteins, because the ratio of solvent-exposed H-bonds to buried H-bonds must then affect the overall stability in a critical manner. The large size of the desolvation penalty suggests that burial of peptide H-bonds must be coupled to a process that provides a favorable free energy change, most likely burial of nonpolar surface area. Coupling has been reported recently (38) between formation of peptide H-bonds and burial of nonpolar surface area when the folding transition state is formed (iii). Side chains larger than alanine hinder the access of water to the helix backbone and substantially reduce the ESF values of peptide groups in a helix (29). Thus, larger nonpolar side chains should give smaller desolvation penalties when H-bonds are buried through folding.

Do calculated ESF values yield the known value for the ΔH of alanine helix formation? The answer to this question is not yet known reliably because it depends critically on the ESF values of peptide groups in the unfolded peptide, which are sensitive both to backbone conformation and to the access of water. Neither is known accurately at present. Until recently, unfolded peptides were assumed to adopt the "random coil" conformation of polymer chemistry, in which there is no preferred backbone conformation and the peptide chain follows a random flight description. However, a recent NMR study of a 7-residue alanine sequence by Neville Kallenbach and co-workers (39) showed surprisingly that the alanine sequence has predominantly the polyproline II backbone conformation. Moreover, the tendency of the unfolded peptide to bend back on itself is important in determining the ESF values of the peptide groups, and little is known about this. I recently compared predicted and observed values for the ΔH of alanine helix formation, based on ESF values, and concluded that they agree within error (30), but the uncertainty associated with ESF values in the unfolded peptide is uncomfortably large.

Some ESF values of a peptide group in structures representative of characteristic stages of protein folding are given for an all alanine peptide in Table I. These ESF values and structures may also be used to consider the process of alanine helix formation. Note that the major uncertainty in representing alanine helix formation lies in the unknown structure of the "unfolded" peptide.

A Paradigm Shift from the H-bond Inventory to Electrostatic Solvation

Adoption of the electrostatic approach to solvation by protein chemists will require a major change in their thinking about peptide H-bonds. The traditional view has been governed by the assumptions of the H-bond inventory in which the H-bonded peptide group does not interact with water, and the net energy of the peptide H-bond depends only on its geometry, not on its exposure to water.

Pressure for change in this traditional view has been building for some time. In 1979 Shneior Lifson and co-workers in their pioneering development of a molecular force field found that the

TABLE I

Predicted ESF values of a peptide group (-NHCO-) in an all alanine peptide with conformations characteristic of stages in the protein folding process

ESF represents electrostatic solvation free energy. A negative value means a stabilizing interaction with the solvent (water), which is treated as a continuum when ESF is calculated with DelPhi and the PARSE parameter set (32). The PARSE parameters (partial charges and atomic radii) are calibrated against experimental values of solvation free energies for a data base of model compounds that includes amides. The structure of a compound must be specified accurately in order to compute its ESF value. This table emphasizes that ESF values depend strongly on backbone conformation as well as on exposure to solvent. The peptide NH and CO dipoles change from being anti-parallel in the extended β-conformation to being parallel in the α_R conformation.

Structure	ESF
	kcal/mol
Solvent-exposed helix[a]	−2.5
Buried helix[b]	0
Extended β-strand (solvent-exposed)[c]	−7.9
Polyproline II (solvent-exposed)[d]	−9.1
Short helix (not H-bonded, solvent-exposed)[e]	−9.5

[a] The ESF value of a fully H-bonded peptide group belonging to residue 8 in the interior of a 15-residue alanine helix (taken from Ref. 29).

[b] The helix is buried out of contact with water. ESF calculations for a variety of structures given in Ref. 37 show that the ESF value drops to zero when exposure of the peptide group to solvent is completely eliminated.

[c] This value, and also the two following values below, represent possible conformations for the unfolded, solvent-exposed alanine peptide. The ESF of a peptide group at an interior position (≥ residue 3) of a solvent-exposed, all-alanine β-strand (ϕ, ψ = −120 °, 120 °) is independent of residue number (taken from Ref. 29). The number of peptide groups equals the number of alanine residues with the numbering scheme used here for peptides with blocked end groups (see Ref. 29).

[d] This value is given for a single alanine residue (number 5) in the polyproline II conformation (ϕ, ψ = −70°, 150°) when all other residues in the 9-residue alanine peptide are in the extended β-strand conformation (taken from unpublished work of F. Avbelj and R. L. Baldwin (manuscript in preparation).

[e] This is a rough value because the ESF value of a peptide group in the α_R conformation (ϕ, ψ = −65°, −40°) depends strongly on the number of neighboring helical residues and on any peptide H-bonds present (see examples in Ref. 29). This value is representative of the first three helical residues of a 5-residue helical sequence with blocked end groups (taken from Ref. 29).

peptide H-bond is represented to a good first approximation by placing dipoles on the peptide NH and CO groups (40) (see Ref. 41 for a recent treatment). If the peptide H-bond can be represented by peptide dipoles, then it is logical to use an electrostatic approach to analyze the solvation free energy associated with the dipoles. In his 1990 review of "dominant forces in protein folding," Ken Dill observed that "transferring a hydrogen bond into a nonpolar medium is generally disfavored" (42), and he gives references to earlier work on the subject. In a 1991 critique of the H-bond inventory, Ben-Naim (31) also discusses the interaction of water with H-bonded polar groups. The desolvation penalty for burying a peptide H-bond was reviewed in 1995 (43) and again later (9) by Barry Honig and co-workers. Modern work on the electrostatic approach to peptide solvation relies particularly on Honig's framework for calculating electrostatic solvation free energy (32) by an algorithm applicable to peptides and proteins.

My own interest in the electrostatic solvation approach was aroused in 1999 with the observation by Peizhi Luo (44) that helix melting curves of peptides differing by only one nonpolar amino acid cross each other at high temperatures. His results indicate that differences in helix propensity among the nonpolar amino acids must be chiefly enthalpic, rather than being entropic as believed. Luo's observation can be explained if nonpolar amino acids with bulky side chains reduce the access of water to the helix backbone (29), as argued earlier by Franc Avbelj and John Moult (45) and elaborated on later by Franc Avbelj (46). A change in solvation of the peptide CO group, when leucine is substituted by alanine, cannot be detected by Fourier-transform infrared measurements (47), however. Avbelj and I found that β-structure propensities, like helix propensities, depend on backbone solvation as judged by a strong correlation between ESF values (37) and unfolding free energies (48) for mutants of a zinc finger protein. Scientists who make electrostatic calculations on proteins generally agree there is a desolvation penalty when polar groups are wholly or partly buried in proteins. Time will tell whether protein chemists will accept that there is a desolvation penalty for burial of peptide H-bonds.

Historical Footnote

I have been asked how I first became interested in this problem. My interest goes back a long time. John Schellman and I became close friends in the 1950s when I was at the University of Wisconsin and he was at the University of Minnesota. As I studied John's classic paper (3) predicting the factors that should control the stability of a peptide helix in water, I often pondered the question: is the α-helix stable in water or not? When our laboratory took up the study of the mechanism of protein folding in 1970, a central question was whether the peptide H-bond is sufficient to stabilize an isolated secondary structure such as an α-helix. In 1970 it was commonly believed that the answer was known, namely that the peptide H-bond cannot by itself stabilize a helix in water because studies of peptides from helical segments of myoglobin (49) and staphylococcal nuclease (50) failed to detect any helix formation. Moreover, helix propensities and helix nucleation constants measured in a host-guest system (51), using a non-natural amino acid as the host (hydroxypropyl- or hydroxybutyl-L-glutamine), gave results indicating that the peptide H-bond will not stabilize a short peptide helix in water. There was, however, a different result reported in 1971 by James Brown and Werner Klee (52), who found marginal helix formation (but only near 0 °C) for the N-terminal helix of ribonuclease A studied in the "C-peptide," which contains the first 13 residues of RNase A. That the C-peptide does indeed form some helix near 0 °C was confirmed in 1982 by Andrzej Bierzynski and Peter Kim in our laboratory (53), and we then began a search for the factors stabilizing this anomalous C-peptide helix. Today many peptides from helical segments of proteins are known to show some helix formation in water.

The search for the origin of helix stability in C-peptide, which was begun both in our laboratory and that of Professor Manuel Rico in Madrid, uncovered one interaction that had been seen in the structure of RNase S (the Glu-2^{-}···Arg-10^{+} salt bridge) and another interaction that was visible in the RNase S structure but not recognized (the Phe-8···His 12^{+} amino-aromatic interaction). The search also found the interaction between the helix dipole and a charged group at either end of the helix, which can be either stabilizing or destabilizing. Finally in 1989 the search led to the discovery by Susan Marqusee (17) that alanine by itself forms a stable peptide helix, although no other natural amino acid has this ability. Therefore, the peptide H-bond must be sufficient to stabilize a helix in water, because very little nonpolar surface area is buried in an alanine helix. The stabilizing effect of the peptide H-bond is still disputed, and it has been proposed (28) that the charged residues needed to solubilize an alanine helix are instead responsible for its stability, although uninterrupted alanine sequences as long as 9 residues (44) or even 13 residues (20) are found to have the high helix propensity characteristic of alanine.

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Address correspondence to: bbaldwin@cmgm.stanford.edu.

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