Annu. Rev. Biophys. Biomol. Struct. 1992. 21:95–118

THE MECHANISM OF α-HELIX FORMATION BY PEPTIDES

J. Martin Scholtz and Robert L. Baldwin

Department of Biochemistry, Stanford University Medical School, Stanford, California 94305–5307

KEY WORDS: α-helix propensities, side-chain interactions in peptides, α-helix stability

CONTENTS

1056–8700/92/0610–0095$02.00

PERSPECTIVES AND SCOPE

The systematic study of helix formation by peptides of defined length and sequence is less than 10 years old. The field began with an effort to understand helix formation in water by an apparently exceptional short peptide, the 13-residue C-peptide from the N terminus of ribonuclease A (RNase A). At that time, other short peptides, with fewer than 20 residues, generally failed to show observable helix formation, and host-guest studies indicated that all short peptides should not show any measurable helix formation if they obey the same rules as random-sequence copolymers. One explanation for the contradiction was that the C-peptide helix might be stabilized by specific side-chain interactions because these should have little effect on helix stability in random sequence copolymers. Amino acid substitution experiments in C-peptide confirmed this explanation and demonstrated that specific side-chain interactions, such as ion-pair and charge-helix dipole interactions, can stabilize short helices in water.

Unexpectedly, however, substitution experiments sometimes revealed large changes in helicity, both in C-peptide and other peptide systems. Moreover, investigators found that they could design short peptide sequences that show good α-helix formation in water, first by using ion-pair interactions to stabilize the helix and later by making use of the unexpectedly strong helix-forming tendency of alanine. These results brought into question the accepted values of helix propensities of the different amino acids, and also raised questions about limitations on the validity of the Zimm-Bragg model of α-helix formation.

The field of peptide helix formation is now at an exciting but speculative stage. Many basic questions are unanswered, but the tools needed to answer these questions are now available. The long-range goal is to understand the mechanism of α-helix formation in water to help elucidate the mechanism of protein folding. A specific goal is to predict the helicity of any arbitrary peptide sequence and further to predict the pattern of helicity residue by residue.

The scope of our review is limited to studies of the past 10 years on α-helix formation in water by peptides of defined length and sequence. We summarize earlier work as background. Insistence on water as the solvent follows from the aim of elucidating the folding mechanisms of water-soluble proteins because they are unfolded by organic solvents. Peptides whose sequences are derived from proteins were the first systems studied. The direction of the field changed when it became possible to study helices formed by sequences of de novo design. This development allowed the isolation and analysis of individual factors in helix formation. Our review

closes by considering the implications of peptide helix studies for the mechanism of protein folding.

HISTORY

A Thermodynamic Model of α-Helix Formation

In 1955, four years after Pauling and coworkers (56) proposed the α-helix as a basic structural motif in proteins, Schellman (68) estimated the stability of the α-helix in water, based on a model still debated today. His model preceded by a few years the 1959 paper by Kauzmann (29) on the hydrophobic interaction. The hydrophobic interaction does not appear in Schellman's model, which assumes that the α-helix is stabilized by the peptide hydrogen bond and destabilized by the loss in backbone conformational entropy. Thus, the free energy change per residue for helix formation was first separated into an enthalpy and an entropy term. In modern usage, *residue* means an α-carbon flanked on both sides by peptide linkages (see below):

$$\Delta G^\circ_{res} = \Delta H^\circ_{res} - T\Delta S^\circ_{res}. \qquad 1.$$

The term ΔH°_{res}, estimated to be -1.5 kcal/mol from data on the dimerization of urea in water (67), was identified with the enthalpy of peptide hydrogen-bond formation in water. The entropy change per residue for helix formation, ΔS°_{res}, was taken to be $-R\ln j$, where j is the number of equivalent torsional conformations of the peptide backbone, per residue, in the random coil form. If j is about 10, ΔS°_{res} is about -4.6 eu and $T\Delta S^\circ_{res}$ is about -1.4 kcal/mol at 25°C. Thus, Schellman (68) concluded that an isolated α-helix should be marginally stable in water.

His formulation of the problem showed the importance of answering the following questions. (*a*) What is the enthalpy of peptide hydrogen-bond formation in water, and what is the value of ΔH°_{res}? (*b*) What is the actual value of j, and how much do side chains contribute to ΔH°_{res}? (*c*) How does the hydrophobic interaction affect the values of ΔH°_{res} and ΔS°_{res}? Given the basic nature of this problem, it is surprising that firm answers to these questions were not obtained long ago. The difficulties resulted chiefly from using polypeptides to study helix formation in water. The use of short peptides with defined sequences allows one to bypass some of these difficulties.

The standard free energy change of forming a helix with n residues is a linear function of ΔH°_{res} and ΔS°_{res}, but it is not proportional to n. One of the two end effects (68) is taken into account by using the modern definition of a residue (see above). The ϕ,φ backbone angles are unconstrained by helix formation in each of the two end amino acids of an unblocked

peptide. These two end amino acids are not, however, counted as residues unless the N and C termini have blocking groups that create two additional peptide units. The second end effect is that the first four NH groups of an unblocked peptide do not form hydrogen bonds in an α-helix, and neither do the four CO groups at the C terminus. Therefore, we must subtract an enthalpy term corresponding to four peptide hydrogen bonds, but the counting system is based on residues (n), not amino acids ($n+2$). The net result is that the term $2\Delta H^\circ_{res}$ needs to be subtracted. Thus, the standard free energy of helix formation by an unblocked peptide, using the modern definition of a residue, is:

$$\Delta G^\circ_{helix} = (n-2)\Delta H^\circ_{res} - nT\Delta S^\circ_{res} \qquad 2.$$

The end effects make formation of a short helix difficult and cause helix stability to depend strongly on chain length. Note that when acetyl and amide blocking groups are present at the N and C termini, they form peptide bonds, contribute two additional peptide units, and increase n, the number of residues, by 2.

Statistical Mechanical Models of α-Helix Formation

Several similar theories of α-helix formation based on statistical mechanics appeared in 1958 and afterwards, and Schellman's model was used to treat the problem with statistical thermodynamics (69). These theories are presented and discussed in the book by Poland & Scheraga (58); the textbook by Cantor & Schimmel (11) gives a clear introduction to the subject. In statistical mechanics treatments, helix formation is a two-step process: helix nucleation can occur at random locations, and helix propagation can take place only after a helical nucleus has been formed. Thus, the process of forming an α-helix is quite different from those biophysical processes that occur in a definite sequence of steps at defined sites, and with unique intermediates. Often with a short peptide, one cannot drive α-helix formation to completion in the conditions available. Because the only possible unique product is the complete helix, the products of the reaction comprise a broad distribution of partly helical molecules with frayed ends. This behavior is predicted by helix-coil theory and is confirmed by current experiments. One basic consequence is that the two-state model (random coil $\rightleftharpoons$ complete helix) is normally a poor approximation to use in treating problems of α-helix formation.

Current practice is to report the parameters of the Zimm-Bragg theory (91, 92) even if another, similar, theory has been used to evaluate the experimental data. These parameters are σ, the helix nucleation parameter, and s, the helix propagation parameter. In the Zimm-Bragg theory, the first helical residue (i), which initiates a helix, is given statistical weight σs

and the second helical residue $(i+1)$ is given statistical weight s. The original Zimm-Bragg theory (92) defined the chain length as the number of peptide units that, in the notation used here, is $n+1$ for an unblocked peptide.

The basic assumption made in applying the Zimm-Bragg model to helix formation by a peptide containing different amino acids is that a single value of s can be used for each type of residue: each value of s is independent of neighboring amino acids and also of the amino acid's position in the helix. The nucleation parameter σ is usually thought to depend on the conformational properties of the peptide backbone in the random coil conformation; thus σ should have similar values for residues with similar ϕ,φ maps (excluding glycine and proline). The intrinsic helix-forming tendency of a residue, or its helix propensity, is assumed to be measured by its value of s, the helix propagation parameter.

The assumption that s is independent of neighboring amino acids is an oversimplification for charged amino acids, which form specific ion-pair interactions with each other and which interact nonspecifically through coulombic interactions. Likewise, the s value of a charged residue depends on its position in the helix through the charge-helix dipole interaction. Several workers have studied the problem of taking account of specific charge interactions while retaining the Zimm-Bragg formalism, which is reviewed briefly below. Nonspecific interactions of an uncharged residue with neighboring side chains may also be significant: this problem is being studied (S. Padmanabhan & R. L. Baldwin, unpublished data).

In the Lifson-Roig model (34) of α-helix formation, a residue is counted as helical or nonhelical according to its ϕ,φ values, in contrast to the Zimm-Bragg model in which it is helical if its peptide NH group is hydrogen bonded. Qian & Schellman (60) discuss the difference between the two models, and also current usage of terms such as residue, in a forthcoming article. The Lifson-Roig theory is particularly useful in studies of helix formation by short peptides because it gives positional information on helix formation: the helicity of each residue can be predicted, once values have been assigned to the nucleation (v) and propagation (w) parameters for each type of residue. Although the v and w parameters of the Lifson-Roig theory differ from the σ and s parameters of the Zimm-Bragg theory, σ and s may be computed readily from v and w by using relations derived by Qian & Schellman (60).

Tests of Helix-Coil Theory and Values for Parameters

In 1959, Zimm et al (93) tested the Zimm-Bragg theory by fitting the helix-coil transitions of a set of polypeptides with different average chain lengths

(26–1500 residues). The peptides were polymers of γ-benzyl-L-glutamate, which does not ionize, and the solvent system was a mixture of a helix-breaker, dichloroacetic acid, and a helix-former, ethylene dichloride. The thermal transition curves for the entire set of polypeptides were fitted satisfactorily by three parameters: σ (assumed independent of temperature), s (at 25°C), and ΔH° (assumed independent of temperature). The value they obtained for s was 2×10^{-4}.

The dependence of s on temperature is written as:

$$-RT \ln s = \Delta H^\circ - T\Delta S^\circ, \qquad 3.$$

where ΔH° and ΔS° are temperature independent. The enthalpy change ΔH° can be identified with ΔH°_{res} in Equation 1, which is measured using calorimetry. Later, the value of ΔH° found by Zimm et al (93) was confirmed using calorimetry (1). The transition curve of the infinite-chain polypeptide gives directly the temperature at which $s = 1$ as the temperature midpoint (T_m) of the unfolding transition, and the shape of the transition curve depends in a simple way on σ and ΔH°. The van't Hoff enthalpy change, ΔH°_{vH}, calculated for a two-state reaction (helix $\rightleftharpoons$ coil) of an infinite-chain polypeptide is given by (2):

$$\Delta H^\circ_{vH} = \Delta H^\circ / \sigma^{1/2}, \qquad 4.$$

where ΔH° is the calorimetrically determined enthalpy change per residue, which appears in Equation 3. The ratio $\Delta H^\circ_{vH}/\Delta H^\circ$ gives the size of the cooperative unit, or the average number of residues in a helical segment at T_m. If $\sigma = 10^{-4}$, the cooperative unit is 100 residues.

The helix-coil transition of γ-benzyl-L-glutamate is inverted in the solvent system studied by Zimm et al (93): the helix is formed at high temperatures. This unusual behavior results from preferential binding by the peptide of one of the two solvent components, and from a change in preferential binding on helix formation (6).

The agreement between helix-coil theory and experiment was excellent in this first studied system (93), which employed a nonionizing amino acid and an organic solvent system. Little further work has been done on testing the theory in systems of this kind, although other properties of the helix-coil transition, such as hydrodynamic properties, have been studied extensively.

The next problem was to study α-helix formation in water. The basic difficulty was that helix-forming amino acids such as Ala and Met yield polypeptides that are not water-soluble, whereas amino acids whose polypeptides are water-soluble, such as Ser, Thr, Asn, Gln, Asp, His, and Arg, do not form the α-helix in water. Only Glu and Lys show helix formation

in water, and then only under special conditions. The uncharged amino acid forms the helix and the ionized form does not, but the uncharged helix aggregates. Zimm & Rice (94) analyzed theoretically the problem of removing charge effects by extrapolation in 1960. They obtained a solution that proved to be quite general (see 49) and widely applicable, and the extrapolations were experimentally feasible. The value of ΔH° (Equation 3) for both Glu and Lys was found to be close to -1 kcal/mol residue (for review, see 59). Rialdi & Hermans (61) measured ΔH° for α-helix formation by Glu with calorimetry and confirmed the value found using the Zimm-Rice method. A value of $\sigma = 0.0025$ was found both for Glu and Lys (59).

Host-Guest Studies

Scheraga and coworkers have used the host-guest method (13, 79, 85, 88) to obtain values of s for all 20 amino acids in the genetic code. Random copolymers of two amino acids are made using the Leuchs synthesis: the host residue is hydroxybutyl- or hydroxypropyl-L-glutamine (HBLG or HPLG) (35), and the guest residue is any of the 20 amino acids. The copolymer is water-soluble and nonionizing unless the guest residue ionizes. Helix-coil transition curves are analyzed using a theory for random-sequence copolymers. A basic assumption of the host-guest method is that the copolymer sequences are truly random. Deviations from randomness seriously affect the shape of the transition curve. For this reason, extracting the value of σ for the guest residue is difficult, and the temperature dependence of s should also be regarded with caution.

The host-guest values of s at 20°C for all 20 amino acids have been determined (88). They show several striking properties. Most values of s cluster closely around 1 ($\pm 20\%$). A value of $s = 1$ means that an amino acid is neither a strong helix-former nor a helix-breaker, but rather is helix-indifferent. The host-guest values for s in water differ strikingly from observations of helix formation by polypeptides in nonpolar solvents, which indicate that β-branched amino acids are helix-breakers (7): Ile shows one of the highest s values found by the host-guest method. The nonionized form of an acidic or basic amino acid always has a higher s value than the ionized form: this is particularly evident for Glu. The temperature dependences of s found using the host-guest method are striking chiefly because of their variability from one amino acid to the next.

Serious contradictions arise between the host-guest values of s and the results of experiments on helix formation with short peptides. These contradictions are discussed in the section below on helix formation by peptides of de novo design.

HELIX FORMATION BY SEQUENCES FROM PROTEINS

Early Studies

In 1968–1971, peptide sequences from helical regions of three proteins were studied. Epand & Scheraga (16) found no significant helix formation at 25°C in peptides from myoglobin, and Taniuchi & Anfinsen (81) reported similar results for staphylococcal nuclease. On the other hand, Brown & Klee (10 and references therein) did find evidence from circular dichroism (CD) studies that the C-peptide (residues 1–13) and the S-peptide (residues 1–20) of ribonuclease A (RNase A) show partial helix formation at low temperatures. Residues 3–13 form a helix in native RNase A, when helical residues are counted as ones that have helical hydrogen bonds. The CD-detected structure in C-peptide corresponds to only about 25% helix at 0°C; it undergoes thermal unfolding so that its helicity is small at 25°C. Brown & Klee found using sedimentation equilibrium that C-peptide is monomeric in conditions in which helical structure is detected. When they used guanidium chloride (GuHCl) to induce helix unfolding, they obtained puzzling CD spectra.

The problem of whether or not C-peptide forms an α-helix in water was reinvestigated by Bierzynski et al (5) in 1982, following a report, using NMR, that structure can be detected in S-peptide (8) at 10°C. Their NMR results showed that residues at well-separated positions participate in structure formation, and the structure undergoes cooperative thermal unfolding. Their CD spectra were consistent with α-helix formation.

pH Dependence of Helix Formation by C Peptide

Helix formation by C-peptide contradicts the host-guest values of s, which predict that no short peptide should show measurable α-helix formation in water (5). The authors used an approximate form of the Zimm-Bragg equation that is not suitable for values of s close to 1; however, a later calculation (75) using the full equation reached a similar conclusion. The stability of the C-peptide helix was found to depend strongly on pH: helix content follows a bell-shaped curve with a maximum near pH 5 (5). At least two ionized groups, one with a pK_a near 3.5 and one with a pK_a near 6.5, are needed for maximum helical stability of C-peptide (5); this observation suggested that specific side-chain interactions stabilize the C-peptide helix and that they explain the contradiction with the host-guest results. A $Glu9^- \cdots His12^+$ ion pair was suggested. The helix content and thermal stability of the C-peptide helix change in parallel as the pH varies, and later studies showed the same parallelism in amino acid substitution experiments.

Helix Stop in S Peptide

The average length of α-helices in proteins is about 11 residues, whereas the average length of a helical segment in a synthetic polypeptide, containing a single type of amino acid, is estimated to be 30–100 residues at T_m (see Equation 4). Thus, proteins have helix termination signals of some kind. Rico and coworkers (63) and, independently, Kim & Baldwin (31) used NMR to study the localization of the helix in S-peptide. Both groups concluded that the helix stops near Met13, and consequently that the helix is localized in S-peptide in a way that resembles helix localization in RNase A.

These investigations provided the first good evidence that studies of α-helix formation in peptides might yield useful information about the mechanism of protein folding. Because helix localization in S-peptide can be explained by helix propensities only if the differences between the *s* values of different amino acids are large, either some specific side-chain interaction acts as a helix stop signal in S-peptide or else the host-guest values of *s* are not applicable to helix formation in S-peptide.

In later studies, a trifluoroethanol (TFE)-H_2O solvent system was used to find out if the helix propagates to the end of S-peptide in the presence of TFE (50); in another study, Asp14, Ser15, and Ser16 were each replaced by Ala (42). Neither study found helix propagation to the C terminus of S-peptide. By providing a fixed nucleus to initiate the helix in S-peptide, Pease et al (57) succeeded in increasing substantially its helix content, and in demonstrating that the helix can be detected four to five residues beyond Met13.

Charge-Helix Dipole Interactions

Chemical synthesis of C-peptide analogs and pH titration of their helix contents (75) showed that $Glu2^-$ and $His12^+$ are the two ionized groups needed for maximal stability of the C-peptide helix. Chemical modification studies (62) pointed earlier to the involvement of $Glu2^-$. Substitution studies confirmed that $Glu2^-$ and $His12^+$, which are far apart in the helix, act independently of each other. Each might interact with the helix dipole because they are close to ends of the helix.

The possibility that charge-helix dipole interactions might affect the stability of the C-peptide helix was tested by Shoemaker et al (76), who used chemical synthesis to vary the charge on the N-terminal residue from $+2$ to -1. They found substantial changes in helix content, and NaCl screening experiments indicated that a charge-helix dipole interaction was responsible. Important experiments from this same period by Ooi and coworkers on the charge-helix dipole interaction are discussed below under peptides of de novo design.

A later study (18) compared the effects on helix stability of the α-NH_3^+ group and the α-COO^- group, and asked if the increase in helix stability found by removing the charge (either with a chemical blocking group or by pH titration) resulted from a charge-helix dipole interaction or from hydrogen bonding. A $-OCH_3$ group, which cannot hydrogen bond to a peptide CO group, was used to block the α-COO^- group; the same increase in helix stability was found as with a $-NH_2$ blocking group, which can hydrogen bond. The results fit the helix dipole model and showed that hydrogen bonding is not involved. If all charge-helix dipole interactions involved hydrogen bonding of the charged side chain to a free peptide NH or CO group, helix-destabilizing interactions should not occur.

The term "charge-helix dipole" interaction may be misleading. The interaction occurs chiefly between the charged group and partial charges on non-hydrogen-bonded groups at either end of the helix (four free NH groups at the N terminus and four free CO groups at the C terminus). Tidor & Karplus (82) recently analyzed the interaction with free energy perturbation calculations for the case of a His → Arg mutation near the C terminus of a protein helix, and Åquist et al (3) made the same point using electrostatic theory.

Phe8 · · · His12$^+$ Interaction in C Peptide

The demonstration (75) that His12$^+$ is one of two charged residues that stabilize the C-peptide helix provoked interest in the mechanism by which His$^+$ acts because: (*a*) His12$^+$ is close to the C terminus of the helix and might stabilize it by interacting with the helix dipole; (*b*) in the X-ray structure of RNase A, the rings of Phe8 and His12 are close together and might interact; (*c*) in host-guest studies (88), His$^+$ has a lower *s* value (i.e. is more helix-breaking) than His^0, and His12$^+$ has to overcome this intrinsic effect of a low helix propensity to stabilize the C-peptide helix. Substitution experiments, combined with pH titration of helix stability, resolved the puzzle (74). They showed that: (*a*) the helix-stabilizing effect results from a Phe · · · His interaction; (*b*) it is specific for an *i*,*i*+4 spacing of Phe and His, and (*c*) the interaction is specific for His$^+$ and vanishes when His12$^+$ is titrated to give His^0. The results also confirmed the earlier puzzling observation that the helix-stabilizing effect of His12$^+$ cannot be screened using NaCl, in contrast to the charge-helix dipole interactions studied earlier.

A current study of the Phe8 · · · His12$^+$ interaction (R. Fairman, K. M. Armstrong & R. L. Baldwin, unpublished results) shows that the above properties are almost unchanged when the Phe-His residues are transferred into an alanine-based peptide with a simple sequence. This study also

suggests that His^+ stabilizes the helix by interacting with the helix dipole, since the helix-stabilizing effect of the Phe-His pair is observed when His is close to the C terminus of the peptide, but not when it is in the center of the peptide.

A molecular dynamics simulation of the C-peptide helix by Tirado-Rives & Jorgensen (83) does not show an interaction between Phe8 and $His12^+$ but does show $His12^+$ in a favorable conformation for interacting with the helix dipole.

$Glu2^- \cdots Arg10^+$ Interaction in C Peptide

The problem posed by identifying $Glu2^-$ as one of the two charged residues that stabilize the C-peptide helix (75) is similar to the problem posed by $His12^+$: does $Glu2^-$ exert its effect by interacting with the helix dipole (since $Glu2^-$ is close to the N terminus of the helix) or by an ion-pair interaction with $Arg10^+$, which can be seen in the X-ray structure of RNase A? The $Glu2^- \cdots Arg10^+$ interaction produces a small kink in the RNase A helix near the N terminus. This kinked conformation was detected using NMR in solution in a study by Osterhout et al (53), who observed an unusual nuclear Overhauser effect (NOE) that is absent if the helix is straight. They also observed a second helical conformation of C-peptide, a straight, extended helix.

The putative helix-stabilizing effect of $Arg10^+$ cannot be demonstrated directly using pH titration because the pK_a of arginine is too high. Substitution experiments (19) gave complex results, probably because the helix straightens out and becomes extended when Ala is substituted for Arg10. By combining substitution experiments with NaCl titrations, however, Fairman et al (19) demonstrated the helix-stabilizing effect of $Arg10^+$. When the substitution $X \rightarrow Ala$ is made and NaCl titrations of helix stability are performed, the curves of helicity versus [NaCl] are nearly parallel for the two peptides with X or with Ala if residue X does not affect helix stability by an electrostatic interaction. If X does interact, and the interaction can be screened with NaCl, then the two curves are nonparallel, and the extent of the deviation from parallelism measures the strength of the interaction.

A molecular-dynamics simulation of the C-peptide helix (83) shows the $Glu2^- \cdots Arg10^+$ ion-pair interaction, but it is a solvent-separated ion pair rather than the contact ion pair seen in the X-ray structure of RNase A.

Studies of Helix Propensities in C Peptide

Initially, investigators conducted substitution experiments in C-peptide (45, 77) to find out if the replacement of a single noninteracting residue

can measurably affect the helicity of C-peptide, and to compare the results with expectations based on the host-guest values of *s*. The results showed that single amino acid substitutions do affect C-peptide helicity, although substitutions made at the N- or C-terminal residue show little effect (77), and the behavior of Ala → Gly (77) or Ala → Ser (45) substitutions fit expectation based on earlier host-guest studies. A later study of Ala → Pro substitutions (78) showed that insertion of a proline residue effectively terminates the C-peptide helix.

Substitution experiments to analyze the Phe8 $\cdots$ His12$^+$ (74) and Glu2$^-$ $\cdots$ Arg10$^+$ (19) interactions sometimes revealed surprisingly large changes in peptide helicity in control experiments, in which the substitution should not have affected any known specific interaction. Later, the explanation for these surprising results was sought through substitution experiments in peptides with simple sequences.

To study systematically the effects of helix propensities on substitution experiments in C-peptide, Fairman et al (17) made the same substitution (Ala → X) at each of three positions in C-peptide (Ala4,5,6) using five different amino acids as X (Glu, His, Phe, Lys, and Arg). They made pH titrations of helicity to aid in sorting out charge interactions and to obtain data on the uncharged forms of Glu and His as well as on their charged forms. The surprising result of this study is that position 5 has a general position effect. Replacement of Ala5 by any of the other five amino acids generally results in a higher helix content in the substituted peptide than when Ala4 or Ala6 is replaced. The reason for this position effect is not known.

The Nascent Helix

NMR study of an immunogenic peptide, corresponding to one helix (residues 69–87) of myohemerythrin, by Dyson et al (15) revealed that residues in the C-terminal half of the peptide have backbone ϕ,φ angles that correspond to the α-helical conformation, although medium-range NOEs characteristic of the helix could not be detected, nor could the helix be observed using CD. Upon addition of TFE, the C-terminal half of the peptide became helical. Consequently, its conformation in aqueous solution was deduced to be a precursor to the helix, and was termed the nascent helix (15). The observation that antibodies made against peptides often cross-react with intact proteins has increased interest in the analysis of peptide conformations in aqueous solution. Dyson et al (14) also found that a single β-turn can be populated sufficiently in a short peptide in water for its conformation to be analyzed using NMR and for the sequence requirements for its formation to be determined. Wright et al (89) reviewed

peptide conformations in water and their analysis using NMR, as well as their implications for the initiation of protein folding.

HELIX FORMATION IN PEPTIDES OF DE NOVO DESIGN

Introduction

The studies of α-helix formation in peptides derived from RNase A identified specific side-chain and charge-dipole interactions that affect the stability of the α-helix. The C- and S-peptide derivatives of RNase A, are not, however, ideal systems for these studies. To isolate and study specific interactions and to measure intrinsic helix-forming tendencies of all of the amino acids, a simple model peptide system is desired. Several independent investigations of α-helix formation in short peptides of de novo design have been made using different model peptides as hosts and different design strategies.

The ideal host peptide should show monomolecular α-helix formation, and its transition from helix to coil must be reversible. An ideal host peptide should, if possible, contain only neutral residues so as to avoid complications from charge-dipole and charge-charge interactions. The stability of the peptide host should derive exclusively from the properties of the polypeptide backbone itself; interactions between side-chain residues should be avoided.

Marqusec & Baldwin (41) described the first de novo-designed α-helical peptide system. It has some of the desirable properties necessary for a simple α-helical host. The peptides in this system mostly contain Ala, with Glu and Lys inserted for solubility. The N- and C-terminal residues are blocked with acetyl and carboxamide, respectively, to eliminate unfavorable charge-dipole effects. The peptides contain oppositely charged residues spaced either $i,i+3$ or $i,i+4$; stabilizing side-chain interactions have been observed for the $i,i+4$ spacing but not for the $i,i+3$ spacing. These peptides, which exhibit $\sim 75\%$ helical structure, demonstrate that simple peptides can be designed and characterized and that specific interactions between side chains can be observed.

This initial success in designing a simple, α-helical peptide led to the design of other helical peptides. Lyu et al (37) synthesized two peptides that contain primarily Glu and Lys; they found modest α-helical structure in solution. These peptides do not contain many Ala residues; rather, the helical structure results solely from ion-pair formation between appropriately spaced Glu and Lys residues (see section below on helix-stabilizing side-chain interactions). Wang et al (87) reported recently that a 285-residue peptide from the smooth muscle protein caldesmon apparently

forms a single long helix and contains numerous pairs of Glu-Lys residues spaced $i,i+4$.

Bradley et al (9) took a contrasting design approach in their studies of another set of monomeric α-helical peptides. They intentionally incorporated many different types of residues to facilitate the NMR assignments. They used NMR to find evidence for an unequal distribution of α-helical structure throughout the chain.

Marqusee et al (43) provided the first direct demonstration of the high helix-forming tendency of alanine. Peptides that contain only Ala, plus either Glu or Lys for solubility, show substantial α-helix formation in water. Because no stabilizing side-chain interactions occur in these peptides, the helical structure results exclusively from the high helix propensity of alanine. Another example of the high helix-forming tendency of alanine is provided by a completely neutral, water-soluble peptide, containing Ala and Gln residues, which exhibits α-helix formation in water (72). This completely neutral peptide, devoid of stabilizing side-chain interactions, meets almost all criteria for an ideal host peptide. With this neutral host, the effects of single charged residues on helix stability, as well as the interactions between charged side chains, can be studied in isolation.

Several attempts have been successful at designing peptides whosc α-helical structure in water is stabilized either by covalent or noncovalent interactions between side chains. Felix et al (20) and Madison et al (40) described analogs of human growth hormone-releasing factor (GRF) that contain a covalent linkage between Asp8 and Lys12 in the helical portion of GRF. These covalently cross-linked analogs show enhanced α-helical structure in solution as well as increased biological activity. Ösapay & Taylor (52) employed the same design strategy in their design of an amphiphilic α-helical peptide containing three pairs of covalently linked Glu-Lys residues. The peptide containing cross-linked Glu-Lys pairs did not, however, show substantially greater α-helical structure than the control peptide with Glu-Lys ion pairs. A recent report by Jackson et al (28) describes the use of single disulfide bond-bridging residues i and $i+8$ to stabilize α-helical structure in peptides as short as eight amino acids. They demonstrate that a D and L pair of isomers of a cysteine homolog, when oxidized to form an intramolecular disulfide bond, can stabilizc the α-helicity of the resulting peptide.

Peptide-metal complexes have also been used to stabilize α-helices. Ghadiri and coworkers have designed peptides that contain two histidines, which can serve as exchange-labile ligands for various transition metals (23) or can form an exchange-inert complex with Ru(III) (24). In the latter case, a 17-residue peptide-metal complex exhibits about 80% α-helical structure in water at 21°C. Ruan et al (66) reported a similar approach to

metal-stabilized peptide helices. In their design, which employs an unnatural amino acid with an aminodiacetic acid side chain, a substantial increase in peptide helicity could be observed in the presence of any of several transition metals.

Helix Propensities Studied in Substitution Experiments

The remarkable success in designing and characterizing short peptides that exhibit substantial α-helix formation in water has prompted a renewed interest in determining the helix-forming tendencies of all the amino acids. Several different approaches have been used, and different host peptides employed, and the results are all qualitatively similar. They show large differences between helix propensities for the various amino acids, larger than those found from host-guest studies (88). The α-helix propensities determined in different systems are, however, quantitatively different; these differences are discussed below. Some of the experiments yield helix propensities measured by values of s; others yield only the change in helix content compared with the host peptide.

Padmanabhan et al (55) provided the relative helix-forming tendencies of the nonpolar amino acids. Marqusee et al (43) describe the host peptide for these studies; X gives the position of an amino acid substitution:

Ac-Y-K-A-A-X-A-K-A-A-X-A-K-A-A-X-A-K-(NH_2).

The reference peptide is devoid of stabilizing side-chain interactions and thus the α-helical structure is stabilized solely by the intrinsic helix-forming tendencies of the constituent amino acids. Of the nonpolar residues studied, Ala, Leu, Ile, Phe, and Val, only Leu shows α-helix formation comparable to Ala. The results do not correlate with the s values determined in host-guest studies (88).

A direct demonstration of the disparity between the s values determined in host-guest studies (88) and those determined in short, alanine-based peptides came from the work of Chakrabartty et al (12). By studying the position-dependent effect of a single Ala → Gly substitution in an Ala-Lys host peptide used earlier (43, 55), and by analyzing the results with the Lifson-Roig theory (34), they found a large ratio of the s values for Ala : Gly, approximately 100. This ratio is in striking contrast to the host-guest ratio of s for Ala : Gly, which is only 1.8 (88). The effect of an amino acid substitution on helix content depends strongly on its position only if the ratio of s values is large. The effect arises from fraying of the ends of the helix.

Merutka et al (44, 46) provided a comprehensive investigation of the helix-forming tendencies of all the amino acids. The host peptide for their substitution experiments,

Ac-Y-E-A-A-A-K-E-A-X-A-K-E-A-A-A-K-A-(NH_2),

is the $i,i+4$ E,K peptide designed by Marqusee & Baldwin (41) and contains three $i,i+4$ stabilizing ion pairs between Glu and Lys residues. They substituted, in turn, each of the 20 residues for the central residue, X, in this 17-residue peptide and determined the helix content of each peptide using CD. Their results show that a single substitution, even in a host peptide that has stabilizing side-chain interactions, can dramatically affect the observed helicity of a short, monomeric peptide.

Another study of the helix propensities of several neutral amino acids was reported by Lyu et al (36). The host peptide employed in these studies,

Suc-Y-S-E-E-E-E-K-K-K-K-X-X-X-E-E-E-E-K-K-K-K-(NH_2),

is based on the earlier peptide described by Lyu et al (37); it contains eight Glu and eight Lys residues appropriately spaced to form eight possible ion pairs. The three central residues, X, were used as substitution sites, and the helical content of each peptide was determined by CD. In addition to using CD to measure the average α-helical content of each peptide, Lyu et al (36, 39) used NMR methods to localize the helical structure within the peptide chain. They find evidence for a nonuniform distribution of helical structure within the peptide; the residues at the ends of the chains are much less helical than those in the middle.

A related study of the helix-forming tendencies of the amino acids has been reported by O'Neil & DeGrado (51), who made single substitutions of all 20 amino acids at a surface-exposed position in a dimeric coiled-coil peptide. Although the coiled-coil is not a monomeric α-helix, O'Neil & DeGrado have selected a substitution site that is solvent-exposed and is not in direct contact with the dimerization surface of the coiled-coil. They determined the effect of the guest residue on the stability of the coiled-coil dimer helix using both urea denaturation and the dependence of helix formation on peptide concentration, and then fitted the results to a two-state reaction between helical dimer and random coil monomers in order to calculate free energy changes. Their results are qualitatively similar to the other substitution results discussed above, but not to the host-guest (88) results. They can also be correlated quantitatively with the data obtained by Lyu et al (36), but not with the host-guest results (88), the Chou-Fasman Pα scales, nor with the results of Chakrabartty et al (12) for Ala → Gly.

Padmanabhan & Baldwin (54) and Lyu et al (38) further investigated the effect of side-chain conformational freedom on α-helical stability. As proposed much earlier by Blout (7), nonpolar β-branched amino acids are helix-breaking, whereas nonpolar straight-chain amino acids are helix-

forming. This result indicates that conformational freedom of a side chain is a major factor in determining its helix-stabilizing tendency. This effect can be analyzed, and even predicted, by free energy simulations. Yun et al (90) conducted a study of this kind for the substitution Ala → Pro in an alanine helix.

The contradiction between host-guest values of s and the results found from substitution experiments in short peptides may result from a helix-stabilizing hydrophobic interaction between the host residues, as suggested by Marqusee et al (43). The host residue, either hydroxypropyl- or hydroxybutyl-L-glutamine (HPLG or HBLG) forms a helix in water that, according to Lotan et al (35), is stabilized by hydrophobic interactions among the long alkyl chains of the host. The net effect is that the intrinsic helix-forming tendency of the guest is masked by the guest's perturbing effect on the stability of the host.

Thermodynamics of α-Helix Formation

All short peptide helices studied thus far, except template-initiated helices and ones stabilized by covalent cross-links, show similar thermally induced unfolding. This observation suggests that helix formation is enthalpically driven. The enthalpy change of helix formation should have two components. The temperature-independent component should reflect formation of peptide hydrogen bond and van der Waals contacts, whereas hydrophobic interactions should be reflected in the temperature-dependent portion of ΔH°.

Scholtz et al (70) have measured the enthalpy of helix formation using differential scanning calorimetry (DSC). The peptide they studied,

$$\text{Ac-Y-(A-E-A-A-K-A)}_8\text{-F-(NH}_2\text{)},$$

has 50 residues and blocked N and C termini and is well-suited to studies of the thermodynamics of the helix-coil transition associated with the polypeptide backbone itself.

The value of ΔH° for helix formation is about -1 kcal/mol residue. The breadth of the thermal transition precludes determining the change in heat capacity, ΔC_p, accurately, but ΔC_p does not appear to be large. Analysis of the thermal unfolding curves for the 50-residue peptide, as monitored by either CD or DSC, reveals a van't Hoff enthalpy change for helix formation (ΔH_{vH}) of -11.2 kcal/mol of peptide, whereas the calorimetric enthalpy change is approximately -1 kcal/mol residue or about -50 kcal/mol peptide. It will be important to determine the enthalpy change of helix formation for other peptides to test the generality of this result and also to measure ΔC_p for the transition. The value of ΔH° found for an alanine-based helix is close to the values found using the Zimm-

Rice method for poly-L-glutamate and poly-L-lysine (for review, see 59) and to the value found calorimetrically for poly-L-glutamate (61). Ben-Naim (4) recently proposed that, considering only the polypeptide backbone, a value for $\Delta H^\bullet$ of about -1 kcal/mol residue should be expected for the formation of a solvent-exposed α-helix in water. He suggests that the ability of the peptide CO group to form more than one hydrogen bond is a key factor in determining the value of ΔH°.

Application of Helix-Coil Transition Theory

A fundamental problem in the study of α-helix formation in water, either by short peptides or by polypeptides, is to determine the applicability of helix-coil transition theory. In testing the applicability of the Zimm-Bragg and Lifson-Roig theories, one must avoid side-chain interactions as much as possible and be aware of possible limitations of the simple model in which the helix propensity of each type of amino acid residue is described by a single value of s, independent of neighboring amino acids and of position in the helix.

Scholtz et al (71) took the same approach used by Zimm et al (93) to test the Zimm-Bragg theory for helix formation by poly-γ-benzyl-L-glutamate in a mixed organic solvent. They studied helix formation in water by a series of simple, repeating-sequence, alanine-based peptides of varying chain lengths, using the thermally induced helix-coil transition monitored by CD. The generic formula is

$$\text{Ac-Y-(A-E-A-A-K-A)}_k\text{-F-(NH}_2\text{)} \quad k = 2\text{–}8.$$

They fitted the helix-coil transition curves with three parameters from helix-coil theory (σ, s at 0°C, and ΔH°), but they also needed to express the values of $[\theta]_{222}$, the mean residue ellipticity (222 nm) of the complete helix and random coil, as functions both of temperature and of chain length. Lack of definite values for these spectroscopic parameters limited the accuracy of the results. Nevertheless, the results were encouraging. They found a value for ΔH° (-0.95 kcal/mol residue) in good agreement with the value measured calorimetrically (70), and the value determined for σ of 0.003 agreed satisfactory with the earlier determinations on poly-L-glutamate and poly-L-lysine ($\sigma = 0.0025$) (for review, see 59).

A second determination of σ for a series of peptides of defined sequence and length in water has been done (C. A. Rohl, J. M. Scholtz, E. J. York, J. M. Stewart & R. L. Baldwin, in preparation). This study, which probes α-helix formation by using amide proton exchange and NMR, determined

a value for σ of 0.0019 for the series of peptides:

$$\text{Ac-(A-A-K-A-A)}_k\text{-Y-(NH}_2\text{)} \quad k = 1\text{–}10.$$

The agreement between these two independent determinations of σ, using two different probes to monitor α-helix formation, suggests that σ may be relatively independent of side-chain type, although further work in this area is needed to resolve this issue.

Studies of Helix-Stabilizing Side-Chain Interactions

Marqusee & Baldwin (41) reported the first observation of ion-pair formation in designed peptides. They used pH and NaCl titrations to investigate the effects of spacing and orientation of putative ion pairs and found evidence for $i,i+4$ but not for $i,i+3$ ion pairs. They also found greater helix stabilization by $Glu^- \cdots Lys^+$ than by $Lys^+ \cdots Glu^-$ ion pairs. Lyu et al's (37) pH titration of the initial host peptide, which contains eight possible $Glu^- \cdots Lys^+$ ion pairs, shows that the helix becomes unstable when the Lys^+ residues are titrated to Lys^0 at pH 12, but not when the Glu^- residues are titrated to Glu^0 at pH 2. This result suggests that singly charged $Glu^0 \cdots Lys^+$ hydrogen bonds may contribute to α-helix stability, as suggested by Marqusee & Baldwin (41).

Merutka & Stellwagen (47, 48) studied the relative effectiveness of several different ion pairs in stabilizing an alanine-based α-helix. They studied Glu-Lys, Glu-Orn, and Glu-Arg ion pairs as well as ion pairs of Asp with each of these three basic residues. Their results indicate that $i,i+4$ ion pairs of all these types can stabilize α-helix formation; differences in helix content were attributed chiefly to the different intrinsic helix-forming tendencies of the various charged residues. The length dependence of helicity was also investigated for the most helical sequences of the peptides studied (48), the $i,i+4$ Glu-Arg peptide. These authors found that increasing the length of the peptide to 27 residues gives a peptide that appears to be completely α-helical under optimal helix-forming conditions.

The problem of quantitating specific side-chain interactions in α-helices is only beginning to be solved. A hierarchical nesting approach has been put forward recently by Robert (64) for incorporating side-chain interactions into the Zimm-Bragg model. He uses existing data in the literature to show how side-chain interactions can be evaluated. Earlier, Vasquez & Scheraga (84) had also proposed a formalism for including side-chain interactions in the Zimm-Bragg model. Gans et al (22) propose a related formalism and use it to evaluate data in the literature. They show that a single set of parameters cannot reproduce the different results found using various host peptides. Their conclusion suggests that the problem of context dependence is a major unsolved problem: how does the apparent s

value of a residue depend on neighboring amino acids, and what are the reasons for these dependencies?

Gans et al (22) use their formalism to calculate the energetic contribution of $i,i+4$ E,K ion pairs to α-helix stability. The two peptides that are compared are:

Suc-Y-S-E-E-E-E-K-K-K-K-E-E-E-E-K-K-K-K-(NH_2) E_4K_4 and

Suc-Y-S-E-E-K-K-E-E-K-K-E-E-K-K-E-E-K-K-(NH_2) E_2K_2.

By comparing the helix contents of two peptides that differ only in the spacing of eight possible ion pairs, such that one peptide, E_4K_4, can form stabilizing ion pairs and the other, E_2K_2, cannot, Gans et al calculate that ion-pair formation contributes 0.50 kcal/mol to the stability of the α-helix in these peptides. The entire helical structure of E_4K_4 results from ion-pair formation; E_2K_2 shows no α-helical structure in water at neutral pH.

Finkelstein and coworkers (21) recently described a computational approach for estimating the helix contents of a wide range of α-helical peptides. They use the Zimm-Bragg formalism with many additional parameters for specific and nonspecific interactions between side chains and the helix macrodipole. They contend that the s values determined from host-guest studies (88) are sufficient to describe the observed α-helix formation by short peptides, provided one includes the energetic contributions to helix formation from side-chain interactions, interactions with the helix macrodipole, and context- and position-dependent adjustments to the intrinsic helix-forming tendencies of the amino acids. Their complex function requires numerous experimental parameters, several of which are not known precisely.

The other major way that a charged side chain can affect the stability of an α-helical peptide, apart from specific ion-pair formation, is the electrostatic interaction between a charged side chain and the helix macrodipole (25, 26, 86). Ooi and coworkers (27, 80) performed a classic set of experiments, which illustrate the properties of the charge-helix dipole interaction and its importance for α-helix stability. They made double-block copolymers of known block lengths, first of Glu and Ala (27) and later of Lys and Ala (80), with 20 residues in each block. In the first block copolymer of each set, the block of ionizing residues was at the N terminus of the alanine block, and in the second block copolymer it was at the C terminus. Stabilization of the alanine helix was observed whenever a block of oppositely charged residues was close to one pole of the helix macrodipole, and helix destabilization was observed if the block of charged residues was of the same sign as the nearby pole of the helix dipole.

Screening by NaCl and other salts was used to show that the charge-helix dipole interaction is electrostatic and to determine whether the block of charged residues stabilized or destabilized the helix. Although the Glu-Ala experiments (27) were performed in 1982, they were not interpreted as being evidence for a charge-helix dipole interaction until 1989, when the Lys-Ala experiments (80) were published.

The existence of the α-helix macrodipole, even in an uncharged peptide, causes the stability of an α-helix to depend on ionic strength according to the theory of Kirkwood (32) for the interaction between an electrolyte and a dipolar species. By applying his theory to the effects of different salts on the helical stability of a completely neutral Ala-Gln peptide, Scholtz et al (72) estimated the dipole moment of the α-helix to be approximately 3.2 Debye per residue, in agreement with direct measurements by Wada (86).

Template-Nucleated α-Helix Formation

Recently, attempts were made to isolate helix propagation from helix initiation, and also to stabilize α-helices, by using specific nucleation templates. Kemp and coworkers (30) have designed a small organic template for helix nucleation, which contains hydrogen-bond acceptors for the first three amide hydrogens of a peptide chain. By constraining the orientation of the hydrogen bond acceptors, the attached peptide chain can adopt an α-helical structure without having to form a helical nucleus. Their results indicate that very short peptides, only four to six residues, can form α-helical structure when attached to the template. Furthermore, they estimate an *s* value for Ala close to that determined from host-guest studies (88). This conclusion conflicts with the results of substitution studies in short peptides and further work will be required to reconcile the differences (for review, see 33).

Pease et al (57) presented another approach to template-nucleated α-helix formation. A hybrid sequence formed from two naturally occurring peptides, apamin from bee venom and the S-peptide from RNase A, was synthesized. In this hybrid peptide, the structure of apamin, which is stabilized by two disulfide bridges, provides a stable α-helical nucleus. Using NMR methods to analyze secondary structure in the S-peptide moiety, Pease et al found substantially more α-helical structure in the hybrid than is observed in the free S-peptide.

Helices that contain either the synthetic nucleus provided by Kemp et al (30) or the natural helical nucleus of Pease et al (57) unfold only gradually by heating or by urea denaturation. This interesting behavior may be explained if helix formation becomes noncooperative when it is initiated by a permanent helical nucleus. As the size of the cooperative

unit shrinks towards a single residue, the van't Hoff ΔH becomes small, and so does the corresponding quantity that characterizes the breadth of the urea denaturation curve.

CONCLUDING REMARKS

Short, highly helical peptides can now be made with a variety of designed sequences. They will be used for many purposes, because peptides sometimes retain the ligand-binding properties of proteins from which they are derived, and they may act as inhibitors of antigen-antibody reactions or of hormone-receptor interactions, and so forth. Our focus here is on the mechanism of α-helix formation by peptides and its significance for analyzing the mechanism of protein folding.

Two features of the problem stand out. First, the study of helix formation by short peptides provides an easy approach to analyzing specific side-chain interactions in helices and to the measurement of the intrinsic helix-forming tendencies, or helix propensities, of the different amino acids. When helix propensities are measured, qualitatively similar but quantitatively different results are obtained using different host peptides. The reasons for this behavior are being worked out.

The second major feature of peptide helix studies is a close relationship, in some cases, between the properties of an isolated helix and the corresponding helix in the intact protein from which it is derived. The sequence that forms the helix is then said to be an autonomous folding unit (73): it carries within the sequence the instructions needed for its proper folding. The best example at present of an autonomous folding unit is the C-peptide helix from RNase A, which contains helix-stabilizing side-chain interactions that are present in the intact protein; also this helix is localized in the longer S-peptide much as it is localized in RNase A.

These features easily explain the rapid growth of this new field. Ten years from now, reviewing the field promises to be a strenuous undertaking.

ACKNOWLEDGMENT

We thank Drs. Hong Qian and John Schellman for advice and Dr. Neville Kallenbach for a preprint of his paper (22), and our colleagues for their discussion: Drs. Avi Chakrabartty and S. Padmanabhan, and Katherine Armstrong, Beatrice Huyghes-Despointes and Carol Rohl. Our own work in this field has been greatly helped by the support of Eunice York and John Stewart at the University of Colorado Medical School at Denver. Our research has been supported by NIH Grant GM 31475, and J.M.S. is a PHS postdoctoral fellow (GM 13451).

Literature Cited

1. Ackermann, T., Neumann, E. 1967. *Biopolymers* 5: 649–62
2. Applequist, J. 1963. *J. Chem. Phys.* 38: 934–41
3. Åquist, J., Luecke, H., Quiocho, F. A., Warshel, A. 1991. *Proc. Natl. Acad. Sci. USA* 88: 2026–30
4. Ben-Naim, A. 1991. *J. Phys. Chem.* 95: 1437–44
5. Bierzynski, A., Kim, P. S., Baldwin, R. L. 1982. *Proc. Natl. Acad. Sci. USA* 79: 2470–74
6. Bixon, M., Lifson, S. 1966. *Biopolymers* 4: 815–21
7. Blout, E. R. 1962. See Ref. 76a, pp. 275–79
8. Blum, A. D., Smallcombe, S. H., Baldwin, R. L. 1978. *J. Mol. Biol.* 118: 305–16
9. Bradley, E. K., Thomason, J. F., Cohen, F. E., Kosen, P. A., Kuntz, I. D. 1990. *J. Mol. Biol.* 215: 607–22
10. Brown, J. E., Klee, W. A. 1971. *Biochemistry* 10: 470–76
11. Cantor, C. R., Schimmel, P. R. 1980. *Biophysical Chemistry*, Vol. 3. New York: Freeman
12. Chakrabartty, A., Schellman, J. A., Baldwin, R. L. 1991. *Nature* (*London*) 351: 586–88
13. Chou, P. Y., Wells, M., Fasman, G. D. 1972. *Biochemistry* 11: 3028–43
14. Dyson, H. J., Rance, M., Houghten, R. A., Lerner, R. A., Wright, P. E. 1988. *J. Mol. Biol.* 201: 161–200
15. Dyson, H. J., Rance, M., Houghten, R. A., Wright, P. E., Lerner, R. A. 1988. *J. Mol. Biol.* 201: 201–17
16. Epand, R. M., Scheraga, H. A. 1968. *Biochemistry* 7: 2864–2872
17. Fairman, R., Armstrong, K. M., Shoemaker, K. R., York, E. J., Stewart, J. M., Baldwin, R. L. 1991. *J. Mol. Biol.* 221: 1395–1401
18. Fairman, R., Shoemaker, K. R., York, E. J., Stewart, J. M., Baldwin, R. L. 1989. *Proteins Struct. Funct. Genet.* 5: 1–7
19. Fairman, R., Shoemaker, K. R., York, E. J., Stewart, J. M., Baldwin, R. L. 1990. *Biophys. Chem.* 37: 107–19
20. Felix, A. M., Heimer, E. P., Wang, C.-T., Lambros, T. J., Fournier, A., et al. 1988. *Int. J. Pept. Protein Res.* 32: 441–54
21. Finkelstein, A. V., Badretdinov, A. Y., Ptitsyn, O. B. 1991. *Proteins Struct. Funct. Genet.* 10: 287–99
22. Gans, P. J., Lyu, P. C., Manning, M. C., Woody, R. W., Kallenbach, N. R. 1991. *Biopolymers*. Submitted
23. Ghadiri, M. R., Choi, C. 1990. *J. Am. Chem. Soc.* 112: 1630–32
24. Ghadiri, M. R., Fernholz, A. K. 1990. *J. Am. Chem. Soc.* 112: 9633–35
25. Hol, W. G. J. 1985. *Prog. Biophys. Mol. Biol.* 45: 149–95
26. Hol, W. G. J., van Duijnen, P. T., Berendsen, H. J. C. 1978. *Nature* (*London*) 273: 443–46
27. Ihara, S., Ooi, T., Takahashi, S. 1982. *Biopolymers* 21: 131–45
28. Jackson, D. Y., King, D. S., Chmielewski, J., Singh, S., Schultz, P. G. 1991. *J. Am. Chem. Soc.* Submitted
29. Kauzmann, W. 1959. *Adv. Protein Chem.* 14: 1–63
30. Kemp, D. S., Boyd, J. G., Muendel, C. C. 1991. *Nature* (*London*) 352: 451–54
31. Kim, P. S., Baldwin, R. L. 1984. *Nature* (*London*) 307: 329–33
32. Kirkwood, J. G. 1943. In *Proteins, Amino Acids and Peptides*, ed. E. J. Cohn, J. T. Edsall, pp. 276–303. New York: Reinhold
33. Lesk, A. M. 1991. *Nature* (*London*) 352: 379–80
34. Lifson, S., Roig, A. 1961. *J. Chem. Phys.* 34: 1963–74
35. Lotan, N., Yaron, A., Berger, A. 1966. *Biopolymers* 4: 365–68
36. Lyu, P. C., Liff, M. I., Marky, L. A., Kallenbach, N. R. 1990. *Science* 250: 669–73
37. Lyu, P. C., Marky, L. A., Kallenbach, N. R. 1989. *J. Am. Chem. Soc.* 111: 2733–34
38. Lyu, P. C., Sherman, J. C., Chen, A., Kallenbach, N. R. 1991. *Proc. Natl. Acad. Sci. USA* 88: 5317–20
39. Lyu, P. C., Wang, P. C., Liff, M. I., Kallenbach, N. R. 1991. *J. Am. Chem. Soc.* 113: 1014–19
40. Madison, V. S., Fry, D. C., Greeley, D. N., Toome, V., Wegrzynski, B. B., et al. 1990. In *Proceedings of the 11th American Peptide Symposium*, ed. J. E. Rivier, G. R. Marshall, pp. 575–77. Leiden, the Netherlands: ESCOM
41. Marqusee, S., Baldwin, R. L. 1987. *Proc. Natl. Acad. Sci. USA* 84: 8898–8902
42. Marqusee, S., Baldwin, R. L. 1990. In *The Protein Folding Problem*, ed. J. King, L. Gierasch, pp. 85–94. Washington, DC: Am. Assoc. Adv. Sci
43. Marqusee, S., Robbins, V. H., Baldwin, R. L. 1989. *Proc. Natl. Acad. Sci. USA* 86: 5286–90
44. Merutka, G., Lipton, W., Shalongo, W., Park, S.-H., Stellwagen, E. 1990. *Biochemistry* 29: 7511–15
45. Merutka, G., Stellwagen, E. 1989. *Biochemistry* 28: 352–57

46. Merutka, G., Stellwagen, E. 1990. *Biochemistry* 29: 894–98
47. Merutka, G., Stellwagen, E. 1991. *Biochemistry* 30: 1591–94
48. Merutka, G., Stellwagen, E. 1991. *Biochemistry* 30: 4245–48
49. Nagasawa, M., Holtzer, A. 1964. *J. Am. Chem. Soc.* 86: 538–43
50. Nelson, J. W., Kallenbach, N. R. 1986. *Proteins Struct. Funct. Genet.* 1: 211–17
51. O'Neil, K. T., DeGrado, W. F. 1990. *Science* 250: 646–51
52. Ösapay, G., Taylor, J. W. 1990. *J. Am. Chem. Soc.* 112: 6046–51
53. Osterhout, J. J., Baldwin, R. L., York, E. J., Stewart, J. M., Dyson, H. J., Wright, P. E. 1989. *Biochemistry* 28: 7059–64
54. Padmanabhan, S., Baldwin, R. L. 1991. *J. Mol. Biol.* 219: 135–37
55. Padmanabhan, S., Marqusee, S., Ridgeway, T., Laue, T. M., Baldwin, R. L. 1990. *Nature* (*London*) 344: 268–70
56. Pauling, L., Corey, R. B., Branson, H. R. 1951. *Proc. Natl. Acad. Sci. USA* 37: 205–11
57. Pease, J. H. B., Storrs, R. W., Wemmer, D. E. 1990. *Proc. Natl. Acad. Sci. USA* 87: 5643–5647
58. Poland, D., Scheraga, H. A. 1970. *Theory of Helix-Coil Transitions in Biopolymers.* New York: Academic
59. Ptitsyn, O. B. 1972. *Pure Appl. Chem.* 31: 227–44
60. Qian, H., Schellman, J. A. 1992. *J. Phys. Chem.* Submitted
61. Rialdi, G., Hermans, J. Jr. 1966. *J. Am. Chem. Soc.* 88: 5719–20
62. Rico, M., Gallego, E., Santor, J., Bermejo, F. J., Nieto, J. L., Herranz, J. 1984. *Biochem. Biophys. Res. Commun.* 123: 757–63
63. Rico, M., Nieto, J. L., Santor, J., Bermejo, F. J., Herranz, J., Gallego, E. 1983. *FEBS Lett.* 162: 314–19
64. Robert, C. H. 1990. *Biopolymers* 30: 335–47
65. Deleted in proof
66. Ruan, F., Chen, Y., Hopkins, P. B. 1990. *J. Am. Chem. Soc.* 112: 9403–4
67. Schellman, J. A. 1955. *C. R. Trav. Lab. Carlsberg Ser. Chim.* 29: 223–29
68. Schellman, J. A. 1955. *C. R. Trav. Lab. Carlsberg Ser. Chim.* 29: 230–59
69. Schellman, J. A. 1958. *J. Phys. Chem.* 62: 1485–94
70. Scholtz, J. M., Marqusee, S., Baldwin, R. L., York, E. J., Stewart, J. M., et al. 1991. *Proc. Natl. Acad. Sci. USA* 88: 2854–58
71. Scholtz, J. M., Qian, H., York, E. J., Stewart, J. M., Baldwin, R. L. 1991. *Biopolymers.* In press
72. Scholtz, J. M., York, E. J., Stewart, J. M., Baldwin, R. L. 1991. *J. Am. Chem. Soc.* 113: 5102–4
73. Shoemaker, K. R., Fairman, R., Kim, P. S., York, E. J., Stewart, J. M., Baldwin, R. L. 1987. *Cold Spring Harbor Symp. Quant. Biol.* 52: 391–98
74. Shoemaker, K. R., Fairman, R., Schultz, D. A., Robertson, A. D., York, E. J., et al. 1990. *Biopolymers* 29: 1–11
75. Shoemaker, K. R., Kim, P. S., Brems, D. N., Marqusee, S., York, E. J., et al. 1985. *Proc. Natl. Acad. Sci. USA* 82: 2349–53
76. Shoemaker, K. R., Kim, P. S., York, E. J., Stewart, J. M., Baldwin, R. L. 1987. *Nature* 326: 563–67
76a. Stahmann, M. A., ed. 1962. *Polyamino Acids, Polypeptides and Proteins.* Madison: Univ. Wis. Press
77. Strehlow, K. G., Baldwin, R. L. 1989. *Biochemistry* 28: 2130–33
78. Strehlow, K. G., Robertson, A. D., Baldwin, R. L. 1991. *Biochemistry* 30: 5810–14
79. Sueki, M., Lee, S., Powers, S. P., Denton, J. B., Konishi, Y., Scheraga, H. A. 1984. *Macromolecules* 17: 148–55
80. Takahashi, S., Kim, E.-H., Hibino, T., Ooi, T. 1989. *Biopolymers* 28: 995–1009
81. Taniuchi, H., Anfinsen, C. B. 1969. *J. Biol. Chem.* 244: 3864–75
82. Tidor, B., Karplus, M. 1991. *Biochemistry* 30: 3217–28
83. Tirado-Rives, J., Jorgensen, W. L. 1991. *Biochemistry* 30: 3864–71
84. Vasquez, M., Scheraga, H. A. 1988. *Biopolymers* 27: 41–58
85. Von Dreele, P. H., Lotan, N., Ananthanarayanan, V. S., Andreatta, R. H., Poland, D., Scheraga, H. A. 1971. *Macromolecules* 4: 408–17
86. Wada, A. 1976. *Adv. Biophys.* 9: 1–63
87. Wang, C.-L., Chalovich, J. M., Graceffa, P., Lu, R. C., Mabuchi, K., Stafford, W. F. 1991. *J. Biol. Chem.* 266: 13958–63
88. Wójcik, J., Altmann, K.-H., Scheraga, H. A. 1990. *Biopolymers* 30: 121–34
89. Wright, P. E., Dyson, H. J., Lerner, R. A. 1988. *Biochemistry* 27: 7167–75
90. Yun, R. H., Anderson, A., Hermans, J. 1991. *Proteins Struct. Funct. Genet.* 10: 219–28
91. Zimm, B. H. 1962. See Ref 76a, pp. 229–33
92. Zimm, B. H., Bragg, J. K. 1959. *J. Chem. Phys.* 31: 526–35
93. Zimm, B. H., Doty, P., Iso, K. 1959. *Proc. Natl. Acad. Sci. USA* 45: 1601–7
94. Zimm, B. H., Rice, S. A. 1960. *Mol. Phys.* 3: 391–407