A salt bridge stabilizes the helix formed by isolated C-peptide of RNase A

(mechanism of protein folding/side-chain interaction/enthalpy of folding/code for helix formation)

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ABSTRACT C-peptide, which contains the 13 NH$_2$-terminal residues of RNase A, shows partial helix formation in water at low temperature (1°C, pH 5, 0.1 M NaCl), as judged by CD spectra; the helix is formed intramolecularly [Brown, J. E. & Klee, W. A. (1971) Biochemistry 10, 470–476]. We find that helix stability depends strongly on pH: both a protonated histidine (residue 12) and a deprotonated glutamate (residue 9 or 2 or both) are required for optimal stability. This information, together with model building, suggests that the salt bridge Glu-9* ··· His-12* stabilizes the helix. Formation of the helix is enthalpy driven [van’t Hoff ΔH, −16 kcal/mol (1 cal = 4.18 J)] and the helix is not observed above 30°C. Proton NMR data indicate that several side chains adopt specific conformations as the helix is formed. These results have two implications for the mechanism of protein folding. First, they indicate that short α-helices, stabilized by specific side-chain interactions within the helix, can be stable enough in water to function as folding intermediates. Second, they suggest that similar experiments with peptides of controlled amino acid sequence could be used to catalogue the intrahelix interactions that stabilize or destabilize α-helices in aqueous solution. These data might provide the code relating amino acid sequence to the locations of α-helices in proteins.

Short α-helices, of the size range usually found in globular proteins (6–20 residues), are highly unstable in water in the absence of specific stabilizing interactions, according to data obtained with random copolymers by the "host-guest" technique (1). For short helices (n < 20) and for σ ≈ 1, the ratio of helical to nonhelical residues depends on the quantity σ = (n + 1)/n (see Note Added in Proof), where σ is the helix nucleation constant, n is an average stability constant for one residue, and n is the number of H-bonded residues in the helix. Values of σ vary from 0.5 to 1.3 for different amino acid residues at 0°C–60°C, while σ is estimated to be ≈10$^{-4}$ (1), so that the fraction of molecules in helical form is expected to be ≈10$^{-4}$ for a short helix. Most studies of helix formation by protein fragments agree with this deduction. The three cyanogen bromide peptides of sperm whale myoglobin give CD spectra that indicate that very little α-helix is present in water at 25°C, although partly helical spectra are obtained in 95% methanol (2). The conclusion has been drawn that tertiary interactions are needed to stabilize the α-helices of globular proteins in water.

An exception was found by Brown and Klee (3): C-peptide of RNase A has a CD spectrum indicative of partial helix formation in water at 1°C. They found that the helix forms monomolecularly, is unstable at 25°C, and is unstable in deionized water. Molecular weight measurements by sedimentation equilibrium showed that C-peptide is monomeric at concentrations up to 1 mM under conditions in which helix formation occurs (1°C, ionic strength, 0.1 M). Freedom from aggregation at moderate concentration is an important property of C-peptide: other studies of possible helix formation in water by protein fragments typically have been plagued by aggregation. The CD measurements of partial helix formation by C-peptide have been made at low concentrations, in which it is exclusively a monomer, both here and in earlier work (3). C-peptide is an NH$_2$-terminal fragment of RNase A (residues 1–13) obtained by cyanogen bromide cleavage at methionine-13. Its sequence, starting from the NH$_2$-terminal end, is Lys-Glu-Thr-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-HSer(lactone). In native RNase S, residues 3–12 form an α-helix and the NH groups of residues 7–13 are H-bonded in the helix (4).

We have confirmed the observations of Brown and Klee (3) and we report here on properties of the helix-forming reaction. Care must be taken to keep the C-peptide in the lactone form since mild alkaline hydrolysis readily converts it to the carboxylic acid form, which has quite different helix-forming properties (to be discussed elsewhere).

MATERIALS AND METHODS

C-Peptide. Preparation of C-peptide by the cyanogen bromide procedure was carried out as described (3). The cleavage reaction results in the conversion of the methionine residue to either homoserine or homoserine lactone. The two forms of the peptide are designated C-peptide carboxylic acid and C-peptide lactone, respectively. These were separated by ion-exchange chromatography on SP-Sephadex C-55: the sample was applied in 50 mM Na cacodylate buffer, pH 5.4, and eluted with a NaCl gradient of 0–0.8 M. The proportions of the two forms were determined by HPLC. The lactone preparation contained 11% of the carboxylic acid form. The CD spectra of the carboxylic acid form were determined separately (sample > 99% pure) and were used to correct the CD spectra of the lactone. In NMR experiments, the resonances of the lactone and carboxylic acid forms are well resolved at low temperatures for several resonances, including the ones studied here. C-peptide concentrations were determined by the ninhydrin method (5), using leucine as a standard. To minimize interconversion between the two forms of C-peptide, the stock solutions of C-peptide lactone and carboxylic acid were kept at pH 2 and pH 10, respectively.

HPLC. The lactone and carboxylic acid forms of C-peptide were analyzed in a Waters HPLC system (model 273 with a U6K injector, model 730 data module, and model 441 detector) using a reverse-phase column (Waters μBondapak C18) with detection at 214 nm. Separation was done isocratically at 25°C using a mobile phase of 6% isopropanol/10 mM ammonium acetate, pH 5.3.

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CD. Spectra were recorded on a Jasco J-500A spectropolarimeter with a thermostatted cell holder in the laboratory of J. T. Yang (University of California Medical School, San Francisco). The cell path length was 1.0 cm. The C-peptide (lactone) concentration was 30 μM unless stated otherwise. Samples contained 0.1 M NaCl/1 mM Na citrate/1 mM Na phosphate/1 mM Na borate, and pH was adjusted with HCl or NaOH.

NMR. Fourier-transform proton spectra were recorded on a Bruker 360-MHz instrument at the Stanford Magnetic Resonance Facility. Carbon-bound proton resonances were observed in $^2$H$_2$O, using Na 2,2,2-tetadeutero-3-trimethylsilyl propanionate as an internal standard, and NH resonances were observed in H$_2$O using the Redfield 2-1-4 pulse-sequence technique. Corrections were made for the pH dependence of the internal standard signal (6). pH values refer to meter readings, either in $^2$H$_2$O or H$_2$O, and the pK values refer to the observed midpoint of a titration curve. The pK values of amino acid side chains are nearly the same in $^2$H$_2$O and H$_2$O when this procedure is followed (7). Samples contained 0.1 M NaCl/20 mM NaOAc. C-peptide concentrations were 2 mM or less and the spectra showed no significant change when the concentration was reduced five-fold.

RESULTS

Helix Formation at Low Temperatures. The CD spectrum of C-peptide lactone at 1.7°C, pH 5.22, has the shape expected for partial helix formation, with a well-defined minimum at 222 nm (Fig. 1). The spectrum is independent of peptide concentration (Fig. 1B) and measurements are made routinely at 30 μM, which is 30-fold below the threshold for aggregation reported by Brown and Klee (3). The most striking feature of the low-temperature spectra is their strong dependence on pH: [θ]$_{222}$ decreases as the pH is changed on either side of pH 5 (Fig. 1).

The pH-dependence curve of helix formation (as measured by [θ]$_{222}$) is bell shaped, with apparent pK values of 3.3 and 6.5 at 3.1°C (Fig. 2). Although the CD spectrum at high temperature (45°C) shows significant ellipticity at 222 nm, the minimum is shifted to higher wavelengths (225–230 nm), and [θ]$_{222}$ is independent of pH (Fig. 2). The properties of any structure remaining at 45°C are clearly different from those of the helix formed at low temperatures (see NMR data). There are large changes in the CD spectrum of C-peptide when guanidinium chloride is added at 25°C, which has been taken as evidence that some structure remains after the helix is melted out (3).

Temperature Dependence of Helix Formation. The helix melts out rapidly with increasing temperature, both at pH 5.04 and at pH 1.90, and [θ]$_{222}$ is nearly independent of temperature above 30°C (Fig. 3). At 1°C, the fraction helix is still increasing strongly as the temperature is decreased. Brown and Klee (3) used methanol/water and ethylene glycol/water to investigate lower temperatures. They found that [θ]$_{222}$ continued to increase below 0°C without leveling off by −13°C and reached values as large as −15,000 deg·cm$^2$·dmol$^{-1}$.

The fraction of molecules in a helical form at pH 5.04, 1°C, can be estimated as follows. We assume that formation of this short helix is, to a first approximation, a two-state (all-or-none) reaction. Then we assume that the helix is completely melted out at high temperatures and use [θ]$_{222}$ at 45°C as a baseline for 0% helix. This is justified by the fact that the CD value at 45°C is essentially independent of temperature and pH (Figs. 2 and 3). At pH 5, the change in [θ]$_{222}$ between 1°C and 45°C is approximately −6000 deg·cm$^2$·dmol$^{-1}$ (Figs. 2 and 3). Next, we need to know [θ]$_{222}$ for complete helix formation. A value of −25,000 deg·cm$^2$·dmol$^{-1}$ is given by the change in [θ]$_{222}$ when
unfolded S-peptide recombines with folded S-protein (9), if the change is attributed entirely to the 10 peptide groups of the helix formed by residues 3–12. Alternatively, a value of $-28,000 \text{deg cm}^2 \text{mol}^{-1}$ is provided by the $\alpha$-helix basis spectrum compiled from 15 proteins with an average helix length of $\approx 10$ residues (10). We use the average of these two values, $-26,500 \text{deg cm}^2 \text{mol}^{-1}$, as a guideline. We assume that the helix formed by free C-peptide is the same as that formed in RNase S (i.e., 10 helical peptide groups) and multiply the change in $[\theta]_{202}$ between 1°C and 45°C ($-6000 \text{deg cm}^2 \text{residue dmol}^{-1}$) by 13/10 to convert this to $-7800 \text{deg cm}^2 \text{mol}^{-1}$ per helical peptide group. This procedure gives 22% ± 2% helix at pH 3, 1°C, but the actual uncertainty in the estimate is larger than this: at least ±5%.

By subtracting the temperature-independent value of the CD at 45°C and assuming that helix formation is an all-or-none reaction, we can calculate an apparent equilibrium constant as a function of temperature. A plot of $\ln K$ vs. $(1/T)$ is reasonably linear and gives a van’t Hoff enthalpy of $-16 \text{ kcal/mol}$ (1 cal $= 4.18 \text{ J}$) for helix formation.

**NMR Study of Side-Chain Interactions.** If the helix is stabilized by intrahelix side-chain interactions, those side chains should adopt specific conformations in the helix and show changes in chemical shift for side-chain protons beyond $\mathcal{C}_p$. Substantial changes have been observed for the side chains of threonine-3, phenylalanine-8, arginine-10 ($\delta \mathcal{N}$), and histidine-12 and also for the $\beta_\text{CH}_3$ protons of one of the three alanine residues. These resonances have been assigned by spin decoupling (to be discussed elsewhere). Large changes may occur also for other side chains whose resonances have not yet been studied because they are less easily resolved. pH titration curves of the chemical shifts for the $\gamma \mathcal{CH}_3$ protons of threonine-3 and for the $\gamma \mathcal{N}$ protons of phenylalanine-8, at 4°C, 21°C, and 47°C are shown in Fig. 4. The transition between the helical and nonhelical forms of C-peptide is fast on the NMR time scale. For each side chain except histidine-12, the pH titration curve at 4°C is bell-shaped with apparent pK values similar to those obtained by CD (Fig. 2). The bell shape diminishes at 21°C and disappears at 47°C. Consequently, as judged by their pH and temperature dependences, these changes in side-chain conformations are part of the helix-forming reaction. Three of the five major lines of the phenylalanine-8 ring spectrum show substantial changes on helix formation while the other two lines show only small changes, indicating that the group or groups that interact with the phenyl ring are not symmetrically placed with respect to the ring center.

**DISCUSSION**

**Helix Stability and the Mechanism of Protein Folding.** The helix formed by C-peptide lactone in aqueous solution is at least 1000-fold more stable than predicted from host-guest parameters. The helix is formed intramolecularly without the need for tertiary interactions. Evidently specific side-chain interactions in the helix are responsible for its stability. If other peptide fragments of proteins also show partial helix formation in water at low temperatures, this will have important consequences for the mechanism of protein folding.

The present experiments show that isolated short $\alpha$-helices can be sufficiently stable to function as folding intermediates. Two models, in particular, for the pathway of protein folding are being discussed at present. In the "framework" model, the H-bonded secondary structure is formed early in folding and provides a framework for stabilizing tertiary interactions that are made later (11–14). The chief objection to this model has been that short $\alpha$-helices and $\beta$-sheets have been thought to be highly unstable in water, so that folding is likely to follow some different pathway with more stable intermediates (15). In the "modular assembly" model, the folding of a subdomain of a protein proceeds almost to completion once it starts, so that its H-bonded secondary structure is stabilized as soon as it is formed by hydrophobic interactions that closely resemble those in the native structure (16). Our results indicate that the framework model is feasible on energetic grounds. They also make the point that intermediates can be stabilized transiently by side-chain interactions that are not present in the final structure of the native protein.

The experiments reported here may be a prototype for ones that solve experimentally that part of the folding code in which the amino acid sequence specifies the locations of $\alpha$-helices and $\beta$-sheets. Lin (17, 18) has proposed a stereochemical theory for such a first stage in folding. His theory is based on model building with assumed stereochemistry of the side-chain interactions; he assumes that hydrophobic interactions are dominant. Our results show that polar interactions (salt bridges and perhaps also H bonds in which either the donor or acceptor is charged) can be important in helix formation and should therefore be considered in schemes for predicting secondary structure from sequence. Moreover, they indicate that it is possible to determine experimentally the side-chain interactions that stabilize or destabilize an $\alpha$-helix in aqueous solution.

**Helix Stabilization by a Salt Bridge.** The pH titration data of Fig. 2 indicate that a salt bridge stabilizes the C-peptide helix, because both a negatively charged glutamate residue and the positively charged form of histidine-12 (or possibly the $\alpha$-NH$_3^+$ of lysine-1) are required for stable helix formation. The term "salt bridge" denotes here an interaction between oppositely charged groups, without implying whether or not they are H bonded. Of the possible candidates, the Glu-9···His-12 salt bridge is the most likely because model building shows that this salt bridge, which can be strengthened by making a linear H bond, will stabilize one turn of the $\alpha$-helix, thus helping to nucleate it. The pH dependence of helix formation does not arise from minimization of the net charge of C-peptide: $[\theta]_{202}$ decreases when histidine-12 is deprotonated, although this reduces the net charge of the C-peptide (lactone) from +3 to +2.

Comparison with the host–guest factors for helix formation in the absence of specific interactions indicates that side-chain interactions should stabilize the C-peptide helix by $\approx 1000$-fold. It will be necessary to study helix formation by chemically modified C-peptide analogues to check that this is correct and to find out which interactions are responsible for the increased stability. The postulated Glu-9···His-12 salt bridge may be
stronger than indicated by the five-fold decrease in $[\theta]_{322}$ between pH 5 and pH 2 or by the three-fold decrease between pH 5 and pH 8 (Fig. 2). Even when one partner in the interaction is uncharged, the other is still ionized and may be able to form a strong H bond, so that helix formation at pH 2 or pH 8 may still be stabilized in part by a Glu-9···His-12 interaction. A charge-stabilized H bond between the deprotonated glutamic acid side chain and its own backbone NH has been reported in short peptides (8).

A Glu-2···Arg-10* salt bridge has been postulated in RNase S (19) and model building indicates that it can be formed in addition to the Glu-9···His-12* salt bridge in the C-peptide helix. Since a Glu-2···Arg-10* salt bridge should be stable at pH 8 but broken when glutamic acid-2 is protonated at pH 2, the small difference between $[\theta]_{322}$ values at pH 2 and pH 8 (Fig. 2) indicates that this salt bridge makes little if any contribution to the stability of the isolated C-peptide helix.

There have been earlier indications that salt bridges may stabilize $\alpha$-helices. Glutamate is the amino acid residue most commonly found in the $\alpha$-helices of native proteins and there is a good correlation between helix probability and the frequency of $(i, i + 4)$ pairs of oppositely charged residues (20). There also is a strong tendency for nearby, oppositely charged, side chains to point toward each other in proteins (21).

Histidine-12 is part of the active site of RNase S and the Glu-9···His-12* salt bridge is not present in the crystal structure of RNase S. If this salt bridge occurs in a folding intermediate, it has to break at a later stage in folding. Glutamic acid-9 is not invariant in evolution (22), which indicates that the Glu-9···His-12* salt bridge is not required for folding.

Energetically, water-accessible salt bridges are thought to be of minor importance in stabilizing the folded structures of proteins as compared with hydrophobic interactions. However, because the net free energy difference stabilizing a native protein is small [−10 to −15 kcal/mol (23)] and because a salt bridge provides a specific interaction between two groups, salt bridges can play an important role in folding. They are known to stabilize the deoxy over the oxy form of hemoglobin (24). A model-building study of ferredoxin suggests that external salt bridges are responsible for the increased thermal stability of the proteins from thermophilic bacteria as compared with ones from mesophiles (25). A salt bridge has been found between the NH$_2$-terminal and COOH-terminal residues of pancreatic trypsin inhibitor and its strength has been estimated as −1 kcal/mol (26). Ion pairing between guanidinium* and carboxylate* ions is strong enough to detect in bimolecular reactions between model compounds, in which $K$ is ∼0.4 M$^{-1}$ (27, 28).

**Helix Formation is Enthalpy Driven.** The large van't Hoff enthalpy change estimated for C-peptide helix formation is interesting for two reasons. First, it suggests that other peptide fragments of proteins may show perceptible structure formation in aqueous solution when measurements are made near 1°C. Most studies that conclude that protein fragments do not contain significant structure have been done at 25°C. Second, it adds to other evidence suggesting that protein folding is driven by a major change in enthalpy as well as by hydrophobic interactions, which are chiefly entropic and show an unfavorable enthalpy change. For example, the $\beta$-sheet in the pH 1.7 folding intermediate of S-protein (residues 21–124 of RNase A) shows a strongly temperature-dependent stability and melts out by 32°C (29). H-bonded intermediates have been detected early in the folding of RNase A at low temperatures (0–10°C) (30, 31).

If we attribute the enthalpy of helix formation in C-peptide to peptide H-bond formation and assume that there are seven H bonds in the C-peptide helix as in RNase S, this gives an enthalpy of −2 kcal/mol per peptide H bond, which is surprisingly large. Note that van't Hoff enthalpies are often not reliable approximations to actual enthalpy changes in reactions involving protein folding (32). The postulated Glu-9···His-12* salt bridge makes little contribution to $\Delta H$, as judged by comparison of the temperature dependences of helix stability at pH 1.90 and pH 5.04 (Fig. 2). Whether or not the peptide H bond in aqueous solution shows a significant $\Delta H$ value has been studied both by measuring association reactions for model compounds and by calorimetry of proteins. However, the question remains controversial. H-bonded dimer formation by N-methylacetamide in water is almost too weak to measure (33), but the $\Delta H$ value has been given as 0. Stronger dimers, with two amide H bonds, are formed by $\delta$-valerolactam and the $\Delta H$ value has been given as −3 kcal/mol per H bond (34, 35). Technical problems with infrared spectroscopy may account in part for the differences between these two values of $\Delta H$ (35).

Scanning calorimetry studies of the thermal unfolding transitions of proteins show that there is a large enthalpy change favoring folding that cannot be attributed to hydrophobic interactions (23). The host-guest parameters for helix formation in water show variable but consistently much smaller apparent enthalpies (1), possibly because hydrophobic interactions be-

**Fig. 4.** pH dependence of chemical shifts of C-peptide lactone in $^{2}$H$_2$O. (A) $\gamma$CH$_3$ protons of threonine-3 at 47°C (Top), 21°C (Middle), and 4°C (Bottom). (B) Major lines shown by ring protons of phenylalanine-8 at 4°C (Left), 21°C (Middle), and 47°C (Right).
between side chains contribute to the apparent enthalpy. Finally, the enthalpy of combination between (largely) unfolded S-peptide and folded S-protein has been measured calorimetrically as $-23$ kcal/mol at $5^\circ$C, pH 7, 0.3 M NaCl (32): the reaction is enthalpy driven. Comparison with our data suggests that helix formation makes a major contribution to the enthalpy change for combination between S-peptide and S-protein.

**Notes Added in Proof.** The fraction of possible H bonds formed ($\theta$) can be calculated from the Zimm–Bragg equation (3b), which reduces to (36)

$$\theta = \frac{y}{1 + y} \left[ 1 - \frac{2}{(n - 3)(s - 1)} \right],$$

with

$$y = \frac{\alpha T^{-1}}{(s - 1)^2}$$

for $n < 20$, $\sigma = 10^{-4}$.

When the data of Fig. 3 are plotted as $\log K$ against $(1/T)$, by using $-26,500$ deg cm$^2$ dmol$^{-1}$ as the estimated value of $\Delta[\theta]$ for complete helix formation, the van’t Hoff $\Delta H$ at pH 1.90 agrees within experimental error with the more precise value of $-16$ kcal/mol obtained at pH 5.04.

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