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Chemistry

Local structure involving histidine-12 in reduced *S*-sulfonated ribonuclease A detected by proton NMR spectroscopy under folding conditions

(protein folding/early-forming local structures)

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ABSTRACT **The $C^{\epsilon}H$ proton resonance of His-12 of reduced cysteine *S*-sulfonated bovine pancreatic ribonuclease A exhibits a nonlinear temperature dependence of the chemical shift in its ^{1}H-NMR spectrum at an apparent pH of 3.0. At temperatures below *ca.* 35°C, the temperature dependence of the chemical shift of the His-12 $C^{\epsilon}H$ resonance is opposite in sign to those of His-48, His-105, and His-119. At temperatures above *ca.* 35°C, the temperature dependence of the chemical shift of the His-12 $C^{\epsilon}H$ resonance is similar to those of the other three His $C^{\epsilon}H$ resonances. These data indicate the existence of an equilibrium between locally ordered and locally disordered environments of His-12 in the sulfonated protein at temperatures below *ca.* 35°C. The ordered and disordered conformations interconvert at a rate that is fast relative to the ^{1}H-NMR chemical shift time scale—i.e., the locally ordered structure has a lifetime of $<<7$ msec. These results demonstrate that short- and medium-range interactions can define short-lived local structures under conditions of temperature and solution composition at which the native protein structure is stable. Furthermore, they demonstrate the utility of reduced derivatives of disulfide-containing proteins as model systems for the identification of local structures that may play a role as early-forming chain-folding initiation structures.**

Locally ordered structures, believed to be determined primarily by short- and medium-range sequence-specific interactions, are thought to play an important role in the initial stages of protein folding (1–8; unpublished data). Under folding conditions (i.e., under conditions of temperature and solution composition at which the native protein structure is thermodynamically stable), the formation of these local structures is thought (1–8; unpublished data) to represent the first step(s) in the folding mechanism. These chain-folding initiation structures are presumed to limit the conformational space accessible to the protein in the initial stages of folding, thereby directing subsequent folding events. With or without internal rearrangements, these locally ordered structures either grow in size or otherwise merge with other independently initiated structures to form either the native protein structure or metastable intermediates which then fold to the native protein structure. It should be emphasized that chain-folding initiation structures, which may direct subsequent folding processes in the parts of the molecule in which they form, need not be involved in structures that participate in the rate-limiting step in folding to the native structure (9–11) since chain-folding initiation may take place at more than one site along the polypeptide chain (7, 8; unpublished data) and the structure(s) involved in the rate-limiting step may not include all of these early-forming local structures.

The problem of determining the conformations of protein structures that form early in the folding process is complicated by the high cooperativity of the folding mechanism, which makes it difficult to isolate folding intermediates under folding conditions. Several approaches involving isotopic labeling of early-forming structures in kinetic folding experiments have been described (12–14), and complementary information *may* be obtainable from equilibrium studies.

For bovine pancreatic ribonuclease A (RNase A), several studies demonstrate the presence of partially folded equilibrium structures at temperatures and pH values corresponding to the thermal transition region (15–18). Spectroscopic studies also indicate the presence of local structure along the polypeptide chain in thermally (19–22), pH- (23), $LiClO_4$- (10, 11), and guanidine- (24) denatured RNase A. Structures important in the folding mechanism *under folding conditions*, however, may be unstable under these denaturing conditions in which the native protein is destabilized. Furthermore, such denaturing conditions may favor the formation of local structures that actually do not take part in the folding mechanism. An alternative approach to determining the conformations defined by local interactions under folding conditions is to characterize those conformations adopted by proteolytic or synthetic fragments of the protein (unpublished data; refs. 25–31). Such studies of conformations adopted by protein fragments, although potentially of great value in identifying the short- and medium-range interactions that play a role in the initial stages of folding, are limited because they usually have relied on predictive schemes to identify amino acid sequences corresponding to chain-folding initiation sites. In addition, ordered intermediate-sized peptides may not exhibit proton Overhauser effects if their proton dipole correlation times τ are of the order of $1/\omega$, where ω is the proton resonance frequency (32).

In view of the shortcomings of these strategies for identifying chain-folding initiation structures, we are attempting to make use of systems (applicable to the entire polypeptide chain) in which long-range interactions are suppressed while short- and medium-range interactions are relatively unperturbed. For disulfide-containing proteins, one such model system is the reduced protein under folding conditions. Although disulfide bonds do not represent the only long-range interactions in a protein, they generally make a major contribution to the stability of the native protein conformation. Several CD (33–35) and Raman (35, 36) spectroscopic studies indicate that significant amounts of local structure are present in reduced RNase A and in reduced cysteine *S*-blocked derivatives. Immunological studies (37) of reduced and reduced *S*-carboxymethylated RNase A indicated that residues 1–13, 31–79, and 80–124 have small amounts of native structure at 4°C. In this report, we describe ^{1}H-NMR measurements demonstrating the presence of local structure involving His-12 in reduced, *S*-sulfonated RNase A under

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conditions of temperature and solution composition at which the disulfide-intact protein is folded.

EXPERIMENTAL

Bovine pancreatic ribonuclease A (type IIAS, Sigma) was purified by carboxymethylcellulose CM52 (Whatman) ion-exchange chromatography (38), desalted on Amberlite MB-1 anion/cation exchange resin, and lyophilized from 0.1 M acetic acid at a concentration of <10 mg/ml. Sulfonated ribonuclease A ($8SSO_3$-RNase) was prepared from purified RNase A by a procedure described in detail elsewhere (unpublished data). Typically, 100 mg of RNase A was dissolved into 5 ml of a well-stirred solution of 0.1 M Na_2SO_3/0.2 M Tris·HCl/6 M guanidine hydrochloride/3 mM ethylenediaminetetraacetic acid, whose apparent pH was 8.0. The disulfide cleavage reaction was allowed to proceed for 10 min, and then 2 ml of a solution of 50 mM 2-nitro-5-thiosulfobenzoic acid/1.0 M Na_2SO_3, pH 7.5, was added. The solution was stirred for 20–30 min and then diluted to a total volume of 14 ml with 0.1 M acetic acid. The pH was adjusted to 3.0 with glacial acetic acid, and the protein was desalted on a 2.5 × 35 cm Bio-Gel P-6DG (Bio-Rad) column using 0.1 M acetic acid as the eluant. The protein fraction was collected and lyophilized from a solution that had a concentration of <1 mg/ml. This procedure has been shown to sulfonate the four cystine disulfides selectively and completely (unpublished data). $8SSO_3$-RNase prepared by this procedure had the following amino acid composition [it should be noted that cystine is regenerated from *S*-sulfocysteine during acid hydrolysis (39)]: $Asx_{14.7(15)}Thr_{10.2(10)}$-$Ser_{14.2(15)}Glx_{12.3(12)}Pro_{4.1(4)}Gly_{3.1(3)}Ala_{11.7(12)}Cys_{7.8(8)}$-$Val_{9.0(9)}Met_{4.0(4)}Ile_{2.8(3)}Leu_{2.1(2)}Tyr_{6.0(6)}Phe_{3.1(3)}His_{4.0(4)}$-$Lys_{10.5(10)}Arg_{4.3(4)}$. Also, this derivative contained <0.01 mol (total) of free SH and disulfide per mol of protein, as analyzed by methods described elsewhere (40), and was >98% homogeneous when analyzed by Mono-Q (Pharmacia) high-performance anion-exchange chromatography using a linear sodium acetate gradient (0–0.7 M sodium acetate in 20 min at a flow rate of 1 ml/min) in 10 mM Tris acetate buffer at pH 8.0.

RNase A was selectively deuterated to different degrees at the four $C^{\epsilon}H$ positions (often referred to as C-2) by the method of Markley (41) as modified by Bierzynski and Baldwin (24). 2-(*N*-Morpholino)ethanesulfonic acid was used in place of cacodylate buffer (11). The protein was then desalted by using Amberlite MB-1 mixed-bed ion-exchange resin and lyophilized from 0.1 M acetic acid. Part of this selectively deuterated protein was then sulfonated according to the procedure described above, taking care to avoid excessive exposure (i.e., >30 min) to alkaline conditions (pH 8), at which slow His $^1H/^2H$ exchange takes place (41).

^1H-NMR spectra were recorded at 300 MHz on a Brüker WM-300 spectrometer equipped with a temperature-regulated probe. The temperature calibration of the NMR probe was checked routinely with a thermocouple immersed in ^2H_2O and was reproducible to within ±1°C. $8SSO_3$-RNase samples were prepared in ^2H_2O containing 0.1 M d_4-deuteroacetic acid (Aldrich), titrated to pH* 3.00 ± 0.05 with deuterium-substituted NaOH (pH* is the apparent pH in deuterated solutions). Partially $C^{\epsilon}H$-deuterated native RNase A samples in ^2H_2O were adjusted to pH* 3.00 ± 0.05 with minute quantities of deuterium chloride. In studies of the temperature dependence of the spectra, 15–20 min were allowed at each temperature for thermal equilibration. In order to obtain high-resolution spectra, the magnetic field homogeneity was adjusted at 5–10°C intervals, and spectra were typically resolution-enhanced using a Gaussian lineshape transformation (42). In quantitative measurements, the total spin recovery time between *ca.* 60° excitation pulses, 5.7 sec, was sufficiently long to ensure complete relaxation of the protein proton spin system, and data manipulation involved simple exponential smoothing corresponding to 0.1 Hz line broadening. Spectra were referenced externally to a solution (1 mg/ml) of sodium 2,2-dimethyl-2-silapentane-5-sulfonate in ^2H_2O contained in a capillary insert.

RESULTS

At 3°C and pH* 3.0, $8SSO_3$-RNase exhibits three ^1H-NMR resonances in the His $C^{\epsilon}H$ region (Fig. 1) with relative intensities of approximately 2:1:1. These three resonances, labeled in Figs. 1 and 2 as resonance A (8.62 ppm), resonance B (8.60 ppm), and resonance C (8.54 ppm), are assigned to the four His $C^{\epsilon}H$ protons of $8SSO_3$-RNase on the basis of their characteristic chemical shift and sensitivity to slow deuterium exchange. The cited values of the chemical shifts pertain to 3°C. To assign the resonances to specific residues in the amino acid sequence, we compared the areas of the His $C^{\epsilon}H$ resonance in partially $C^{\epsilon}H$-deuterated native RNase A with those of the same deuterated protein after rapid sulfonation. At pH* 3.0 and 25°C, the $C^{\epsilon}H$ resonances of

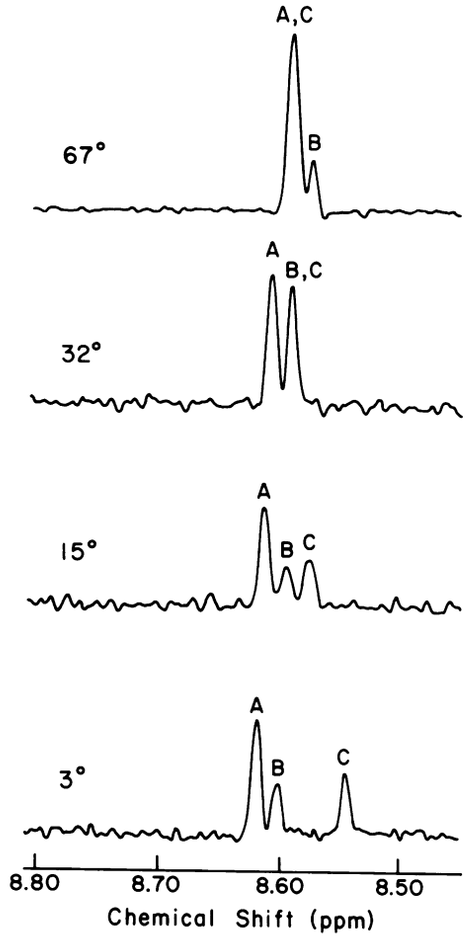

FIG. 1. Temperature dependence of the His $C^{\epsilon}H$ resonances of $8SSO_3$-RNase at a protein concentration of 10 mg/ml in 0.1 M deuteroacetic acid at pH* 3.00. The temperatures of the measurements (in degrees Celsius) are indicated. The reference is external sodium 2,2-dimethyl-2-silapentane-5-sulfonate. Resonance A is assigned to the overlapping resonances of the $C^{\epsilon}H$ protons of His-48 and His-119, resonance B to His-105 $C^{\epsilon}H$, and resonance C to His-12 $C^{\epsilon}H$. These spectra have been resolution enhanced by transformation to a Gaussian free induction decay prior to Fourier transformation (42). At temperatures below *ca.* 35°C, resonances A and B move to higher field, while resonance C moves to lower field, with increasing temperature. At temperatures above *ca.* 35°C, the chemical shift of resonance C coincides with that of resonance A, and all resonances move to higher field with increasing temperature. Similar results were obtained for samples at a protein concentration of 2 mg/ml.

partially deuterated native RNase A exhibited four well-resolved resonances (not shown here) assigned (41) to His-12, His-48, His-105, and His-119 with areas corresponding to 20% ± 1%, 60% ± 2%, 6% ± 1%, and 14% ± 1%, respectively, of the total area of the $C^{\epsilon}H$ resonances. In the partially deuterated and sulfonated protein at pH* 3.0 and 3°C, resonances A and B together accounted for 79% ± 1% of the total area of the $C^{\epsilon}H$ resonances, while resonance C represented the remaining 21% ± 1%. From these measurements, resonance C is assigned specifically to His-12 $C^{\epsilon}H$ because His $^1H/^2H$ exchange is minimal during the short time of exposure to the alkaline conditions (pH ≈ 8) of sulfonation (41) and both the native and sulfonated proteins were therefore deuterated to the same extent. Because resonances A and B overlap considerably *in the absence of resolution enhancement* (spectrum not shown), accurate integration of these two resonances individually was not possible. However, in the resolution-enhanced spectra, the area of resonance B of deuterated $8SSO_3$-RNase A was so much smaller than that of resonance A that resonance B could be assigned unambiguously to His-105 $C^{\epsilon}H$—i.e., in resolution-enhanced spectra, resonances A and B represent 71% and 5%, respectively, of the total area of the $C^{\epsilon}H$ resonances, while the His-105 $C^{\epsilon}H$ resonance of partially deuterated native ribonuclease represents *ca.* 6% (cited above) of the total area of the $C^{\epsilon}H$ resonances. Hence, resonance B is assigned to His-105 $C^{\epsilon}H$ and resonance A to the overlapping $C^{\epsilon}H$ resonances of His-48 and His-119.

In principle, nonequivalence of His $C^{\epsilon}H$ resonance frequencies at a given temperature may be due either to the presence of some amount of locally ordered structure in equilibrium with the ensemble of disordered conformations or to conformation-independent amino acid sequence effects on chemical shift. To distinguish between these, we examined the temperature dependence of the His $C^{\epsilon}H$ chemical shifts between 3°C and 77°C (Figs. 1 and 2). As can be seen in Fig. 2, the chemical shifts of both resonances A (i.e., $C^{\epsilon}H$s of His-48 and His-119) and B (i.e., His-105 $C^{\epsilon}H$) vary linearly with temperature, but the chemical shift of resonance C (i.e., His-12 $C^{\epsilon}H$) exhibits distinctly nonlinear behavior. Below *ca.* 35°C, the chemical shift of His-12 $C^{\epsilon}H$ increased with increasing temperature, while above this temperature it decreased with increasing temperature (Fig. 2). This nonlinear temperature dependence of the chemical shift is attributable to the presence of one or more local structures involving His-12 in $8SSO_3$-RNase, which are in temperature-dependent equilibrium with the ensemble of disordered conformations. At temperatures below *ca.* 35°C, the observed chemical shift of the His-12 $C^{\epsilon}H$ resonance is a population-weighted average of the chemical shifts of ordered and disordered conformations that interconvert at a rate that is rapid relative to the ^1H-NMR chemical shift time scale (see *Discussion*). This conformational equilibrium between structures involving His-12 (at pH* 3.0) is thermally reversible (see legend of Fig. 2), independent of concentration, and independent of the age of the sample stored frozen over a period of 1 month. Above *ca.* 35°C, the chemical shift of His-12 $C^{\epsilon}H$, like those of His-48, His-105, and His-119, decreases linearly with increasing temperature. This high-temperature behavior apparently represents the temperature dependence of the His-12 chemical shift (relative to external sodium, 2,2-dimethyl-2-silapentane-5-sulfonate) for the locally disordered ensemble of conformations, the slope of which is indistinguishable from that of the other three His $C^{\epsilon}H$ resonances above *ca.* 35°C. It should be noted that the linear dependence of the chemical shifts of the His-48, His-105, and His-119 $C^{\epsilon}H$ resonances on temperature over the entire temperature range does not exclude the possibility that these residues may also be involved in locally ordered structures because, unlike those of His-12, their side-chain chemical shifts may possibly be indistinguishable for ordered and disordered backbone conformations.

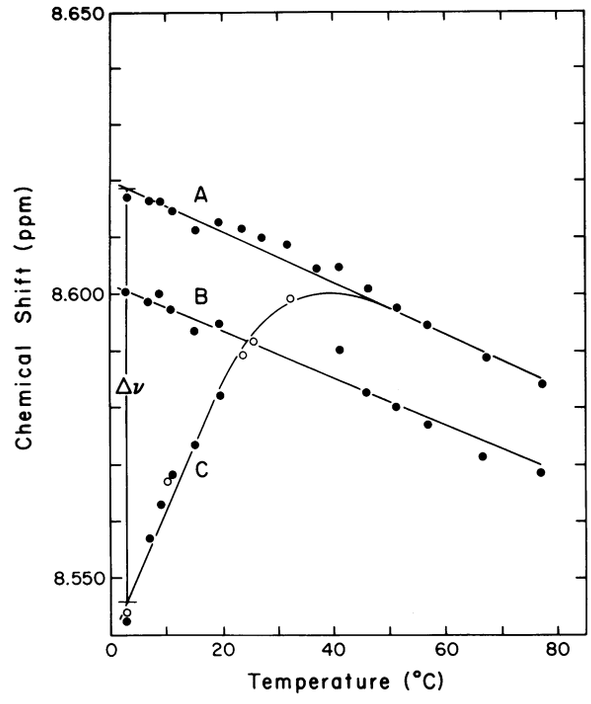

FIG. 2. Temperature dependence of His $C^{\epsilon}H$ resonances of $8SSO_3$-RNase. The solvent conditions and resonance assignments are described in the legend of Fig. 1. The solid circles are data obtained by heating His $C^{\epsilon}H$-protonated $8SSO_3$-RNase from 3°C to 77°C. The open circles are data obtained by first heating (to 47°C) and then cooling a sample of $8SSO_3$-RNase A in which the His-105 $C^{\epsilon}H$ position had been almost completely deuterated. This enabled assignments to be made in the crossover region at *ca.* 25°C. At each temperature in the partially His $C^{\epsilon}H$ deuterated data set, resonance A was assigned a chemical shift value obtained from a linear least-squares fit of the data for resonance A of the fully $C^{\epsilon}H$ protonated protein—i.e., resonance A was used as the internal reference for the open-circle data set. The meaning of $\Delta\nu$ is discussed in the text.

DISCUSSION

These ^1H-NMR results on $8SSO_3$-RNase indicate that, at temperatures less than *ca.* 35°C, the chemical shift of the $C^{\epsilon}H$ proton of His-12 is a population-weighted average resulting from a temperature-dependent equilibrium between the ensemble of disordered conformations and one or more locally ordered structures. At temperatures above *ca.* 35°C, the chemical shift of the $C^{\epsilon}H$ proton of His-12 is identical to those of His-48 and His-119. The temperature dependence of these three resonance frequencies (relative to external sodium 2,2-dimethyl-2-silapentane-5-sulfonate) above *ca.* 35°C therefore appears to correspond to the temperature dependence of the chemical shift for His $C^{\epsilon}H$ protons in the ensemble of locally disordered conformations at pH* 3.0.

We considered the possibility that the upfield shift of the His-12 $C^{\epsilon}H$ resonance at temperatures less than *ca.* 35°C might be due to conformation-independent intrinsic titration shifts (43) arising from a small change in the degree of protonation of either Asp-14 or Glu-9 over the temperature range of the measurements. In the absence of local structure, both aspartic and glutamic acid side chains should be predominantly protonated at pH* 3.0, and the temperature-dependent change in the degree of protonation should be small since ΔH for ionization of carboxyl groups is generally close to zero. Since intrinsic titration shifts for next-nearest or more distant neighbors are also small (43), it follows that such effects should be negligible at pH* 3.0. Indeed, His-48,

which is *adjacent* to Glu-49, exhibits a linear dependence of the $C^{\epsilon}H$ chemical shift on temperature (resonance A of Fig. 2), clearly demonstrating the insignificance of conformation-independent titration shift effects in these measurements.

Over the temperature range of these measurements (3°C to 77°C), the His-105 $C^{\epsilon}H$ resonance was 17.4 ± 0.8 parts per billion (ppb) upfield from the His-48 and His-119 $C^{\epsilon}H$ resonances, although these three resonances all exhibit approximately the same temperature independent *slope* of chemical shift vs. temperature—i.e., −0.44 ± 0.02 ppb/degree. This upfield shift may be attributed either to conformation-independent amino acid sequence effects on chemical shift or to the presence of local structure involving His-105, which is not destabilized over the temperature range studied here.

As the equilibrium between locally ordered and locally disordered environments of His-12 shifted with temperature, the chemical shift of the His-12 $C^{\epsilon}H$ proton varied continuously while the relative areas of the three resolved resonances (i.e., A, B, and C) remained constant. This result, seen clearly in Fig. 1, demonstrates that the locally ordered and locally disordered conformations involving His-12 interconvert at a rate that is fast relative to the proton chemical shift time scale. By extrapolating the linear temperature dependence of the His-12 chemical shift observed above 35°C (corresponding to the temperature dependence of the chemical shift for the locally disordered conformations) to temperatures below 35°C, the frequency difference ($\Delta\nu$) between the chemical shift expected for His-12 $C^{\epsilon}H$ in the absence of local structure (i.e., the extrapolated value) and that observed experimentally can be determined. The maximum observed value of $\Delta\nu$ (see Fig. 2) was *ca.* 23 Hz at 3°C. Presumably, for the fully locally ordered structure (i.e., at temperatures < 3°C) the value of $\Delta\nu$ is even greater. Taking 23 Hz as a *minimum value* of $\Delta\nu$ for the fully locally ordered structure, the *upper limit* for the lifetime of this local structure estimated[‡] from the Heisenberg uncertainty principle is 7 msec (at 3°C)—i.e., the lifetime of the structure must be *much shorter* than 7 msec. Hence, this short-lived structure is both thermodynamically and kinetically accessible under folding conditions and would form very rapidly in a solvent jump from unfolding to folding conditions at pH* 3.0.

In view of the body of evidence demonstrating that both the S-peptide (11, 24, 26, 30, 31) (i.e., residues 1–20) and C-peptide lactone (25, 28) (i.e., the cyanogen bromide cleavage product corresponding to residues 1–13) fragments of RNase A adopt some amount of α-helical backbone structure in water at temperatures less than *ca.* 40°C, our finding that, *under folding conditions*, His-12 of reduced and sulfonated RNase A participates in one or more short-lived local structures (i.e., with a lifetime << 7 msec) suggests that the α-helical backbone conformation is also adopted by these residues (i.e., residues 3–13) in $8SSO_3$-RNase. The upper bound on the lifetime of this structure is consistent with that observed for α-helices of homopolymers (44, 45), which have lifetimes on the order of 10^{-6} sec. The assignment of the structure to an α-helix is also supported by immunochemical studies (37) in which it was found that antibodies directed primarily against the native α-helical conformation of residues 3–13 can be used to detect small amounts[§] of native-like structure at pH 8.3 and 4°C in reduced ($K_{conf} \approx 7 \times 10^{-2}$) and in *S*-carboxymethylated ($K_{conf} \approx 1 \times 10^{-3}$) ribonuclease. Circular dichroic spectroscopic studies also indicate the presence of *ca.* 14% α-helical structure (at pH 6, 25°C) in reduced, carboxyamidomethylated ribonuclease (34), although that study (34) does not provide information on the location in the amino acid sequence of this local helical structure.

The present ^{1}H-NMR study indicates that the local structure involving His-12 of $8SSO_3$-RNase has a His $C^{\epsilon}H$ chemical shift *upfield* from that expected for a disordered ensemble of structures. Examination of the crystal structure (46, 47) of RNase A reveals that, in the native α-helical conformation of residues 3–13, the aromatic ring of Phe-8 is situated directly over the His-12 imidazole ring. In the absence of other major contributions to the chemical shift (e.g., solvent exclusion effects and inter-fragment interactions like those in the folded protein), the resulting ring-current shift of Phe-8 on His-12 is anticipated to cause an upfield shift of the side-chain resonances of His-12, like that observed for the His-12 $C^{\epsilon}H$ resonance of the locally ordered structure of $8SSO_3$-RNase.

Local structures, thought to be α-helices involving His-12, have also been identified in $LiClO_4$ (10, 11)- (pH* 3.0, 25°C, 3.5 M $LiClO_4$) and guanidine hydrochloride (24)- (pH* 1.9, 9.2°C, 1.5–3 M guanidine) denatured, disulfide-intact RNase A. The precise role, if any, of helices involving His-12 in the folding mechanism is not yet clear. The lifetime, τ, of the locally ordered structure reported in this study is much less than 7 msec, much shorter than the fastest phase of the folding of the disulfide-intact protein [$\tau \approx$ 20–400 msec (48–50)]. Therefore, the degree of local structure involving His-12 in early-forming folding intermediates will be determined by a rapidly established equilibrium, due to short- and medium-range interactions, prior to subsequent folding events. Kinetic experiments (11) show that the rate of folding under conditions at which the helix involving His-12 is stabilized is similar to the rate of folding under conditions at which it is destabilized (when these rates are extrapolated to identical final folding conditions). These results indicate that the NH_2-terminal helix does not take part in the rate-limiting step. It is pertinent to note that S-protein, which lacks the 20 NH_2-terminal residues, can fold to a native-like conformation (51, 52) in the absence of S-peptide. In combination with the NMR results presented here, this suggests that early-forming local structures may arise independently in these two parts of the molecule and implies the existence of one or more other chain-folding initiation sites (7, 8; unpublished data) in the S-protein portion of the molecule.

Although a systematic evaluation of the effects of various cysteine S-blocking groups on the stability of local structures containing cysteine is not yet available, the results of this study demonstrate the utility of reduced derivatives of disulfide-containing proteins as model systems that allow the identification of conformations defined by short- and medium-range interactions under folding conditions. The results presented here provide spectroscopic evidence that reduced, sulfonated RNase A has local structure involving His-12 at pH* 3.0 at temperatures less than *ca.* 35°C in aqueous solution. It is not yet known if the presence of disulfide bonds affects the stability of local structures involving His-12, which may form early in the folding of the disulfide-intact protein. The local structure that we report in the present work may, however, be important in the oxidative regeneration of RNase A from the reduced protein. Although immunochemical studies (51) indicate that antibody recognition

[‡] $\tau << 1/(2\pi\Delta\nu)$ for fast exchange (see ref. 32, pp. 116–120), where τ is the lifetime of the individual states.

[§] An antigenic binding site does not necessarily correspond to a simple sequence of amino acids in the native protein, but rather to a distribution of atoms on the surface of the protein, part of which includes those residues identified as the antigenic site by competition studies with peptide fragments. For this reason, one cannot be certain that a given locally ordered amino acid sequence, either in a peptide fragment or in a reduced RNase A derivative, contains all of the information required to form a complete antigenic determinant. The degree of order (expressed in terms of K_{conf}) determined by these immunochemical techniques therefore represents an approximate lower bound for the actual amount of local native structure.

sites of the S-protein (residues 21–124) can be fully regenerated from the reduced S-protein in the absence of S-peptide (residues 1–20), the full native enzymatic activity is not regained when S-peptide is added to regenerated S-protein (53, 54), suggesting (51) a role for residues 1–20 in determining the regenerated conformation(s) of residues 21–124.

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1. Kotelchuck, D. & Scheraga, H. A. (1968) *Proc. Natl. Acad. Sci. USA* **61,** 1163–1170.
2. Kotelchuck, D. & Scheraga, H. A. (1969) *Proc. Natl. Acad. Sci. USA* **62,** 14–21.
3. Scheraga, H. A. (1973) *Pure Appl. Chem.* **36,** 1–8.
4. Wetlaufer, D. B. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 697–701.
5. Tanaka, S. & Scheraga, H. A. (1977) *Macromolecules* **10,** 291–304.
6. Wetlaufer, D. B. & Rose, G. D. (1981) in *Biomolecular Structure, Conformation, Function, and Evolution*, ed. Srinivasan, R. (Pergamon, New York), Vol. 2, pp. 97–101.
7. Matheson, R. R., Jr., & Scheraga, H. A. (1978) *Macromolecules* **11,** 819–829.
8. Némethy, G. & Scheraga, H. A. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 6050–6054.
9. Konishi, Y., Ooi, T. & Scheraga, H. A. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 5734–5738.
10. Denton, J. B., Konishi, Y. & Scheraga, H. A. (1982) *Biochemistry* **21,** 5155–5163.
11. Lynn, R. M., Konishi, Y. & Scheraga, H. A. (1984) *Biochemistry*, in press.
12. Schmid, F. X. & Baldwin, R. L. (1979) *J. Mol. Biol.* **135,** 199–215.
13. Kim, P. S. & Baldwin, R. L. (1980) *Biochemistry* **19,** 6124–6129.
14. Kuwajima, K., Kim, P. S. & Baldwin, R. L. (1983) *Biopolymers* **22,** 59–67.
15. Benz, F. W. & Roberts, G. C. K. (1975) *J. Mol. Biol.* **91,** 345–365.
16. Burgess, A. W. & Scheraga, H. A. (1975) *J. Theor. Biol.* **53,** 403–420.
17. Chen, M. C. & Lord, R. C. (1976) *Biochemistry* **15,** 1889–1897.
18. Gilbert, W. A., Lord, R. C., Petsko, G. A. & Thamann, T. J. (1982) *J. Raman Spectrosc.* **12,** 173–179.
19. Roberts, G. C. K. & Benz, F. W. (1973) *Ann. N.Y. Acad. Sci.* **222,** 130–147.
20. Matthews, C. R. & Westmoreland, D. G. (1975) *Biochemistry* **14,** 4532–4538.
21. Howarth, O. W. (1979) *Biochim. Biophys. Acta* **576,** 163–175.
22. Matthews, C. R. & Froebe, C. L. (1981) *Macromolecules* **14,** 452–453.
23. Labhardt, A. M. (1982) *J. Mol. Biol.* **157,** 331–355.
24. Bierzynski, A. & Baldwin, R. L. (1982) *J. Mol. Biol.* **162,** 173–186.
25. Brown, J. E. & Klee, W. A. (1971) *Biochemistry* **10,** 470–476.
26. Silverman, D. N., Kotelchuck, D., Taylor, G. T. & Scheraga, H. A. (1972) *Arch. Biochem. Biophys.* **150,** 757–766.
27. Stimson, E. R., Montelione, G. T., Meinwald, Y. C., Rudolph, R. K. E. & Scheraga, H. A. (1982) *Biochemistry* **21,** 5252–5262.
28. Bierzynski, A., Kim, P. S. & Baldwin, R. L. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2470–2474.
29. Kim, P. S., Bierzynski, A. & Baldwin, R. L. (1982) *J. Mol. Biol.* **162,** 187–199.
30. Rico, M., Nieto, J. L., Santoro, J., Bermejo, F. J., Herranz, J. & Gallego, E. (1983) *FEBS Lett.* **162,** 314–319.
31. Kuwajima, K. & Baldwin, R. L. (1983) *J. Mol. Biol.* **169,** 281–297.
32. Jardetsky, O. & Roberts, G. C. K. (1981) *NMR in Molecular Biology* (Academic, New York), pp. 63–65.
33. Schaffer, S. W. (1975) *Int. J. Peptide Protein Res.* **7,** 179–184.
34. Takahashi, S., Kontani, T., Yoneda, M. & Ooi, T. (1977) *J. Biochem.* **82,** 1127–1133.
35. Galet, A., Creighton, T. E., Lord, R. C. & Blout, E. R. (1981) *Biochemistry* **20,** 594–601.
36. Lord, R. C. & Relyea, N. M. (1981) *J. Raman. Spectrosc.* **11,** 315–319.
37. Chavez, L. G., Jr., & Scheraga, H. A. (1980) *Biochemistry* **19,** 1005–1012.
38. Taborsky, G. (1959) *J. Biol. Chem.* **234,** 2652–2656.
39. Bailey, J. L. & Cole, R. D. (1959) *J. Biol. Chem.* **234,** 1733–1739.
40. Thannhauser, T. W., Konishi, Y. & Scheraga, H. A. (1984) *Anal. Biochem.* **138,** 181–188.
41. Markley, J. L. (1975) *Biochemistry* **14,** 3546–3554.
42. Ferrige, A. G. & Lindon, J. C. (1978) *J. Magn. Reson.* **31,** 337–340.
43. Bundi, A. & Wüthrich, K. (1979) *Biopolymers* **18,** 299–311.
44. Cummings, A. L. & Eyring, E. M. (1975) *Biopolymers* **14,** 2107–2114.
45. Zana, R. (1975) *Biopolymers* **14,** 2425–2428.
46. Wlodawer, A., Bott, R. & Sjölin, L. (1982) *J. Biol. Chem.* **257,** 1325–1332.
47. Borkakoti, N., Moss, D. S. & Palmer, R. A. (1982) *Acta Crystallogr.* **B38,** 2210–2217.
48. Lin, L.-N. & Brandts, J. F. (1983) *Biochemistry* **22,** 564–573.
49. Tsong, T. Y., Baldwin, R. L. & Elson, E. L. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1809–1812.
50. Tsong, T. Y. & Baldwin, R. L. (1978) *Biopolymers* **17,** 1669–1678.
51. Chavez, L. G., Jr., & Scheraga, H. A. (1980) *Biochemistry* **19,** 996–1004.
52. Labhardt, A. M. & Baldwin, R. L. (1979) *J. Mol. Biol.* **135,** 231–244.
53. Haber, E. & Anfinsen, C. B. (1961) *J. Biol. Chem.* **236,** 422–424.
54. Kato, I. & Anfinsen, C. B. (1969) *J. Biol. Chem.* **244,** 1004–1007.