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Nuclear Magnetic Resonance Evidence for a Structural Intermediate at an Early Stage in the Refolding of Ribonuclease A

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The kinetics of refolding of heat-unfolded ribonuclease A have been studied by Fourier transform proton nuclear magnetic resonance at 10°C, pH 2. A single refolding reaction is observed: it corresponds to the slow-refolding reaction seen in stopped-flow studies of refolding at higher temperatures. There are two results of interest for the mechanism of protein folding. (1) A new resonance (X) is observed that shows the presence of a structural intermediate in refolding. (2) The α-helix close to the N-terminal end of ribonuclease A apparently forms rapidly when the unfolded protein is brought to refolding conditions.

The folding intermediate has been studied by monitoring the C-2 protons of the four histidine residues. The intermediate contains one residue (X) in a partly folded environment and the other three residues in unfolded environments. The composite resonance (U) of these three protons at 10°C agrees with the average chemical shift of the histidine residues in heat-unfolded ribonuclease A at high temperatures. During refolding at 10°C, the resonance intensities of U and X disappear at the same rate that the spectrum of native ribonuclease A is regained.

Partial deuteration experiments show that X is either histidine 12 or 119. Comparative studies of the amino-terminal fragment 1–20 of ribonuclease A indicate that X is histidine 12. The appearance of structure in this peptide can be followed by temperature-dependent changes in the chemical shift of histidine 12. At 10°C the chemical shifts of histidine 12 and X agree closely. These results are consistent with the circular dichroism study of peptide 1–13 by Brown & Klee (1971), who concluded that helix formation occurs at low temperatures.

1. Introduction

Equilibrium studies of the reversible folding reactions of small globular proteins provide little evidence for populated intermediate states on the pathway of folding (for reviews, see Tanford, 1968,1970; Privalov, 1974). On the other hand, recent stopped-flow and temperature-jump studies indicate that kinetic intermediates can be observed in these same folding transitions (for a review, see Baldwin, 1975). Whether or not some of the kinetic intermediates are structural intermediates on the pathway of folding has been an open question. It is clear that one of the most intensively studied intermediates, the species responsible for the fast refolding

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reaction of RNAase A (bovine pancreatic ribonuclease A:EC no. 3.1.4.22), is not a partly folded intermediate but rather a configurationally different form of the unfolded protein (Garel & Baldwin, 1973,1975*a*,*b*; Brandts *et al.*, 1975; Hagerman & Baldwin, 1976; Garel *et al.*, 1976). The fast-refolding and slow-refolding forms of unfolded RNAase A may result from the slow *cis–trans* isomerization of prolyl residues in the polypeptide backbone (Brandts *et al.*, 1975).

Proton nuclear magnetic resonance provides a promising approach for detecting structural intermediates. By using rapid pulsed n.m.r.†, a series of spectra can be obtained at one-minute intervals while the protein is in the process of folding. If partly folded intermediates are present, new resonances may be observed whose chemical shifts are different from those of the native or unfolded forms at equilibrium. At low temperatures, the kinetics of folding become slow enough to measure by proton n.m.r. At 10°C (pH 2) the slow-refolding reaction of RNAase A has a half-life of three minutes.

The C-2 protons of the four histidine residues are well-resolved from other protons in the spectrum of the native protein taken in ^2H_2O. They can be used to study the folding of four segments of the molecule. His12 and His119 are part of the catalytic site of native RNAase A, while His48 is at the hinge of the active site crevice. Both His48 and His105 are in the β-pleated sheet which forms the backbone of the molecule. His12 is in an α-helix (residues 3 to 12 in RNAase S). For the X-ray structures of RNAase A and RNAase S, see Carlisle *et al.* (1974), Wyckoff *et al.* (1970) and Richards & Wyckoff (1973). The C-2 protons of the four histidine residues have already been used to study the equilibrium folding transition of RNAase A (Westmoreland & Matthews, 1973; Matthews & Westmoreland, 1973; Benz & Roberts, 1975). A very convenient property of the C-2 protons is that they exchange slowly with solvent and can be exchanged out in ^2H_2O at mild alkaline pH values at rates which differ for the four histidine residues. This property can be used to assign the resonances corresponding to these protons. Note that the assignments of His12 and His119 have been reversed recently (cf. Patel *et al.*, 1975).

In our experiments RNAase A is first unfolded by heating to 46°C (at pH 2) in an n.m.r. tube, then chilled rapidly in an ice bucket and brought to 10°C in the n.m.r. probe. Fourier transform spectra are recorded at one-minute intervals during refolding. About one-half of the sample has refolded when the first spectrum is recorded. There are two reasons (cf. Hagerman & Baldwin, 1976): (1) unfolded RNAase A contains 20% of a fast-refolding species, which refolds about 100 times more rapidly than the major (80%) slow-refolding species and the folding of this fast-refolding species cannot be studied in our experiments; and (2) at higher temperatures, inside the unfolding transition zone, the rate of the slow-refolding reaction is considerably faster than at 10°C, and part of the sample refolds on cooling through this zone.

2. Materials and Methods

Bovine pancreatic ribonuclease A (Worthington Biochemical Corporation: grade RAF, phosphate free) was further purified by chromatography on CM-Sephadex C-50 with a 0 to 0·3 M-salt gradient (Garel, 1976). Activity was assayed following the method of Crook *et al.* (1960).

† Abbreviations used: n.m.r., nuclear magnetic resonance; RNAase S, RNAase A cleaved between residues 20 and 21; S-peptide, the amino-terminal fragment of RNAase A (residues 1 to 20); p.p.m., parts per million.

RNAase S was obtained from Sigma Chemical Company and treated similarly; S-peptide was prepared by the method of Doscher & Hirs (1967).

Labile protons were replaced by deuterons by heating a 1% solution of RNAase in distilled 99·7% ^2H_2O at 55°C, pH 3·0 for 15 min. The solution was then lyophilized, and exchanged and lyophilized a second time. For most experiments the protein was then dissolved in distilled 99·97% ^2H_2O, 0·2 M-NaCl to a final volume of 0·4 ml. Samples were placed in 5-mm n.m.r. tubes (Wilmad Glass Co., type 507-PP). Unless otherwise specified, solutions were 10% protein by weight ($\sim 10^{-2}$ M). pH is used to refer to a glass electrode meter reading uncorrected for the deuterium isotope effect. pH was adjusted using 1 M and 0·1 M solutions of sodium deuteroxide and deuterium chloride (Wilmad).

The original spectra were obtained on a Varian XL100 spectrometer equipped with a disk accessory. A series of 30 s or 60 s spectra were taken in the pulsed Fourier transform mode using a 1 s pulse repetition rate and a 60° pulse angle. The time-averaged free induction decays were stored on a disk. More recent spectra were taken on a Bruker 360 MHz spectrometer with a Nicolet disk system.

Most temperature-jump experiments were carried out at pH 2·0, where the protein is completely unfolded at 45°C and completely folded at 10°C. Samples of protein were unfolded by heating to 45°C in a constant temperature block. The protein was cooled rapidly in an ice-water bath to 10°C and then placed in the spectrometer probe, previously equilibrated to 10°C. Data acquisition is begun within 30 s after the temperature change. Free induction decays are obtained and stored on the disk while the protein is refolding. After about 25 min, folding is complete, and the free induction decays are transformed, plotted and integrated using a Hewlett Packard 9864A digitizer and 9810A calculator. Reproducibility of area measurements is ±10%.

3. Results

(a) *Nuclear magnetic resonance spectra recorded during refolding at 10°C (pH 2)*

Figure 1 shows 360 MHz spectra of the C-2 protons of the four histidine residues at three different temperatures (all at pH 2·1): at 46°C, where RNAase A is unfolded and a barely resolved doublet is seen; at 10°C, where four different C-2 resonances are seen in the native protein; and at 37°C, inside the transition zone for unfolding, where both sets of resonances are seen. To a first approximation, the spectrum for partial unfolding at equilibrium is a weighted average of the native and unfolded spectra. For discussion of this type of experiment on RNAase A, see Westmoreland & Matthews (1973) and Matthews & Westmoreland (1973). Backbone amide protons that have not been completely exchanged out in ^2H_2O may appear in this region and distort the baseline.

Spectra taken at different times of refolding at pH 2·0, 10°C, are shown in Figure 2. Considerable refolding has occurred by the time the first spectrum is recorded, for reasons given in the Introduction. After 11 minutes the spectrum is essentially the same as that of native RNAase A (Fig. 1). At one minute and at three minutes, two unfolded peaks are observed one of which (U) corresponds to the composite resonance of heat-unfolded RNAase A at 46°C (see Fig. 1). The chemical shift of U measured at high temperatures is practically independent of temperature (Westmoreland & Matthews, 1973), and can be extrapolated down to 10°C. The second resonance X, which is upfield from U, is not observed in the spectra either of the native or of the unfolded forms (Fig. 1) and it disappears with time at about the same rate as U. Thus its kinetic behavior resembles that of the composite unfolded resonance. The proton area of X is always less than unity, and the ratio of areas for U to X is about 3:1.

The change with time of each peak area is shown in Figure 3. About one-half of

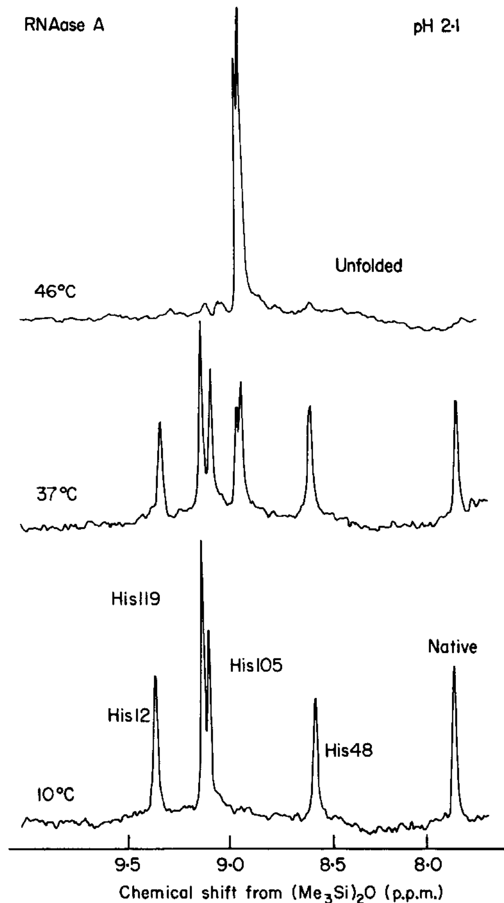


FIG. 1. Spectra at pH 2·1 of the C-2 protons of the 4 His residues of RNAase A in 3 conditions: native (10°C), heat unfolded (46°C), and inside the transition zone for thermal unfolding (37°C). The upfield resonance at 7·9 p.p.m. is the C-4 proton of His105 (Markley, 1975). Conditions: 8 mM-RNAase A, 0·2 M-NaCl in ^2H_2O.

the sample is refolded at zero time. The appearance of the different native resonances and the disappearance of U and X follow essentially the same kinetics. In Figure 3 one exponential curve with the time constant $\tau = 183$ seconds has been used to fit the appearance of the three native peaks and the disappearance of U + X.

In some refolding experiments, the peak at the position of His48 in the native RNAase A spectrum appeared to increase in area at early times more rapidly than the peaks for His12 and for His119+105. This effect could not be reproduced in all experiments. For this reason, and also because of the difficulty in measuring peak areas accurately in kinetic experiments, we leave the resolution of this question for future work.

(b) *Assignment of X: partial deuteration experiments*

The results above suggest that the unfolded protein, at the time of the first n.m.r. spectrum, has been converted to a folding intermediate in which one histidine residue (X) is in a partly folded environment and the other three histidine residues are in unfolded environments. This interpretation can be checked and X can be assigned by experiments in which different histidine residues are selectively deuterated, making

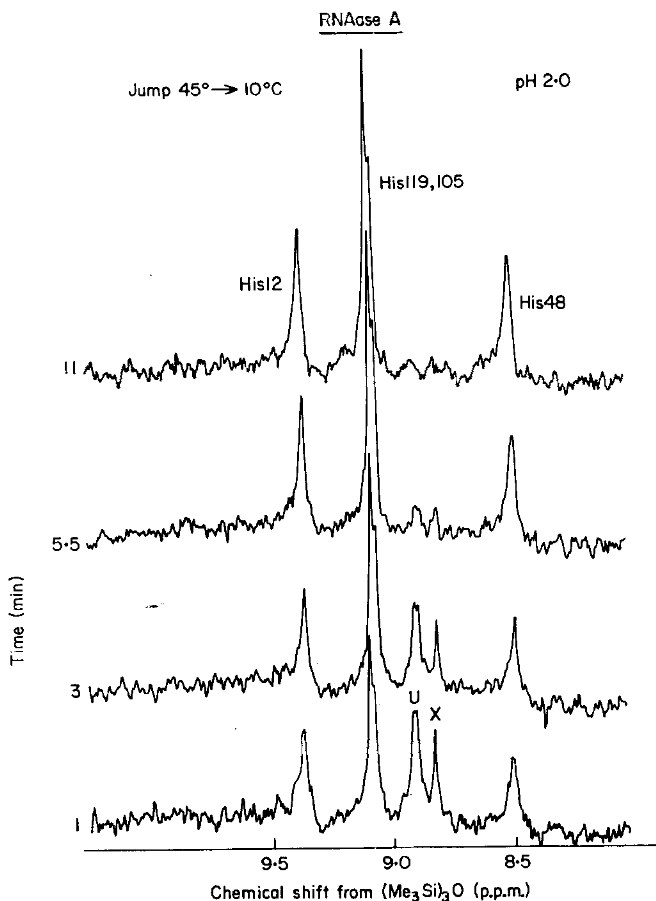


FIG. 2. Spectra recorded during folding at 10°C after rapid cooling of heat-unfolded RNAase A at 45°C. Spectra are shown at 1, 3, 5·5 and 11 min after cooling. Conditions: pH 2·0, 8 mM-RNAase A, 0·2 M-NaCl in ^2H_2O.

use of the different pK values of the four histidine residues and of the inhibition of exchange of His12 and His119 by binding 2′-CMP. Figure 4 shows the refolding kinetics of a sample in which His105 has been extensively deuterated. Resonance X is still present and its peak area relative to that of U has increased. Thus X does appear to be the resonance of a C-2 proton from a single histidine residue, and X is not His105. Figure 5 shows an experiment in which both His12 and His119 are extensively deuterated. Resonance X is absent, showing that X is either His12 or His119.

(c) *Comparison of X with His12 in the S-peptide*

Since the partial deuteration results show that X is either His12 or His119, and since a circular dichroism study by Brown & Klee (1971) shows that the N-terminal peptide 1-13 becomes partly helical at low temperatures, we guessed that X might be His12 and that the partly folded environment of X might be the α-helix containing residues 3 to 12 in RNAase S (Wyckoff *et al.*, 1970). Consequently we compared the chemical shift of X with that of His12 in the S-peptide. Figure 6 shows that they are

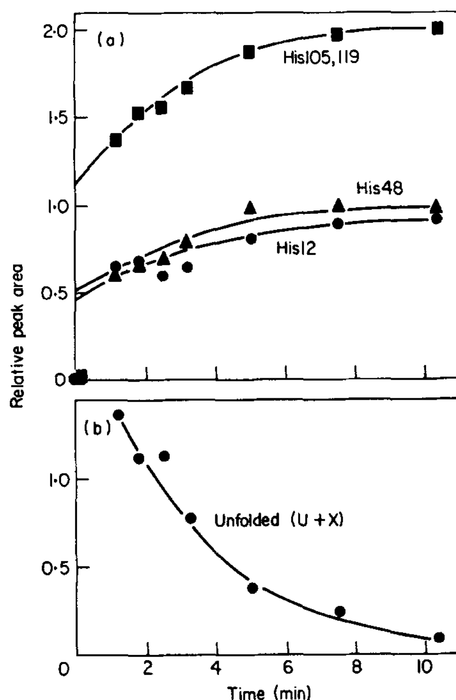


FIG. 3. The kinetics of appearance of individual C-2 protons in native RNAase A (a) and disappearance of the 2 resonances shown by initially unfolded RNAase A (b) after rapid cooling from 45°C to 10°C. This is the same experiment as in Fig. 2.

very similar at 10°C, pH 2. Figure 7 shows that the formation of structure in the S-peptide at temperatures below 30°C can be monitored by changes in the chemical shift of His12. Above 30°C the chemical shift of His12 relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate is independent of temperature and coincides with the upfield resonance of the doublet in heat-unfolded RNAase A (Fig. 1). In refolding at 10°C the unfolded histidine doublet is not resolved; the resonance labelled U in Figure 2 has the average chemical shift of the doublet seen at 46°C (Fig. 1).

4. Discussion

(a) *Formation of the nearly N-terminal α-helix of RNAase A in refolding conditions*

Brown & Klee (1971) demonstrated by circular dichroism that the N-terminal peptide 1–13 of RNAase A becomes partly helical at low temperatures and high salt concentrations. Their measurements were made in the pH range 5 to 6. They found that the helix is not very stable, and is largely melted out at 25°C. They suggested that formation of this helix is an important early step in the folding of RNAase A. Our measurements are made on somewhat different materials (on S-peptide, residues 1 to 20, and also on an unfolded species of RNAase A) and in different conditions (pH 2·0 *versus* pH 5 to 6, 0·2 M-NaCl *versus* 0·033 M-Na_2SO_4), but our S-peptide results appear to be consistent with theirs, and our RNAase A results confirm their postulate that the nearly N-terminal α-helix of RNAase A is an intermediate in folding. Brown & Klee found that peptide 1–13 aggregates at concentrations above 1 mg/ml in their conditions. Most of our measurements on S-peptide are made at

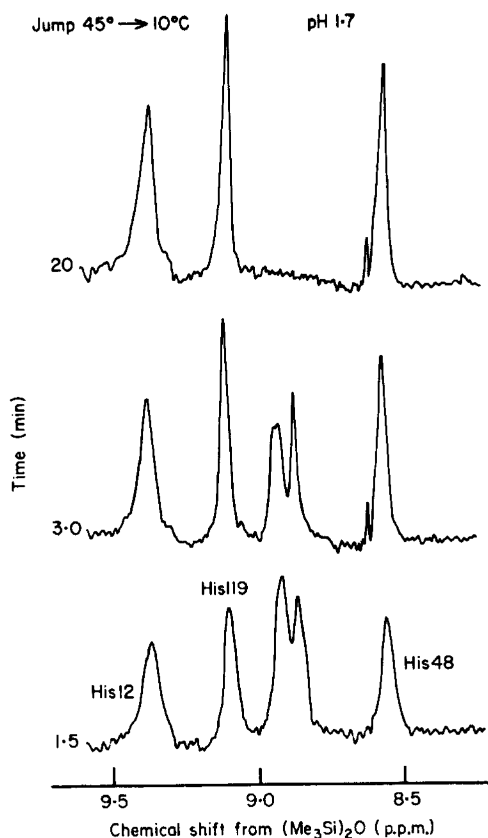


FIG. 4. Kinetics of refolding of an RNAase A sample in which the His105 peak has been much reduced by exchange for 1 week in ^2H_2O in 20 mM-2′-CMP, pH 7·5, 40°C. Spectra are shown at 1·5, 3 and 20 min after rapid cooling from 45°C to 10°C. Conditions: pH 1·7, 8 mM-RNAase A, 0·2 M-NaCl in ^2H_2O.

10 to 20-fold higher concentrations, but at pH 2·0. The line-width of His12 in the S-peptide at pH 2 is quite sharp and is comparable, for example, to that of X in unfolded RNAase A (Fig. 6). Dr A. A. Schreier has repeated these S-peptide spectra at a concentration of 0·5 mg/ml, and has found the same temperature-dependent changes in the chemical shift of His12. (Figure 7, A. A. Schreier, personal communication). The changes in chemical shift can be attributed to fast exchange on the n.m.r. time-scale between two conformations, which presumably are an α-helix and the unfolded S-peptide. In synthetic polypeptides, slow n.m.r. exchange between helix and unfolded peptide has apparently been observed: see the discussion by Miller (1973) of possible mechanisms.

(b) *Significance of the intermediate as regards the mechanism of folding*

The first and most significant finding is that a structural intermediate exists and can be characterized. This probably means that other protein folding reactions also possess intermediates that are stable enough to be populated at least transiently.

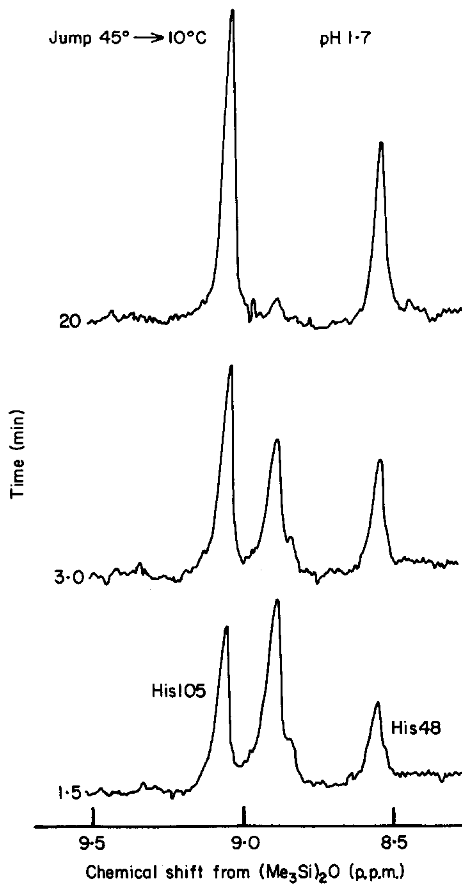
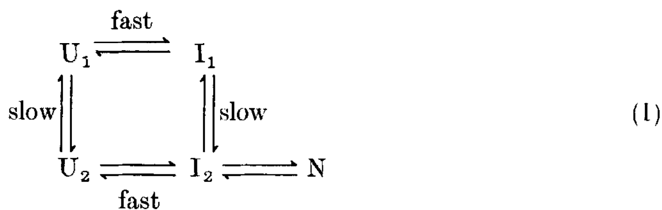


FIG. 5. Kinetics of refolding of an RNAase A sample in which His12 and His119 have been extensively deuterated by exchange in ^2H_2O followed by back-exchange in H_2O for 1 week in 20 mM-2′-CMP, pH 7·5, 40°C. Spectra are shown at 1·5, 3 and 20 min after rapid cooling from 45°C to 10°C. Conditions: pH 1·7, 8 mM-RNAase A, 0·2 M-NaCl in ^2H_2O.

Hawley & Macleod (1976) have found a long-lived kinetic intermediate in the reversible folding transition of chymotrypsinogen A: it can be separated by fast electrophoresis after rapid cooling to 0°C, pH 2. Our second finding is that the folding intermediate apparently contains the helix found near the N-terminal end of native RNAase A.

These findings present the following questions. (1) What other folded structures are contained in the early folding intermediate? (2) What is the position on the pathway of folding of the early folding intermediate? (3) Does the intermediate play a functional role in directing the progress of folding? (4) What bonding interactions stabilize the early folding intermediate?

Some of these questions are now open to study, but little can be said that is definite. As regards question (1), it should be possible in future work to search for other amino acid residues that are in partly folded environments, using different n.m.r. techniques. As regards questions (2) and (3), the accompanying paper (Nall *et al.*, 1978) suggests a pathway for RNAase A folding and places on it an intermediate (I_1) whose properties fit those reported here.



(1)

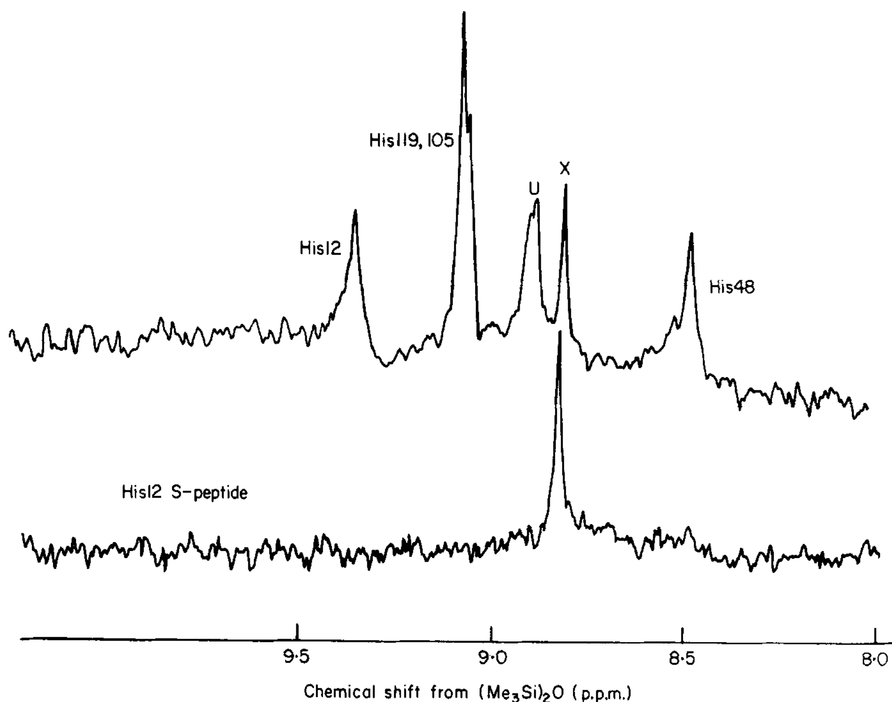


FIG. 6. Comparison between His12 in the S-peptide at pH 2·0, 10°C, and resonance X in the early folding intermediate (spectrum taken at 1 min in Fig. 2).

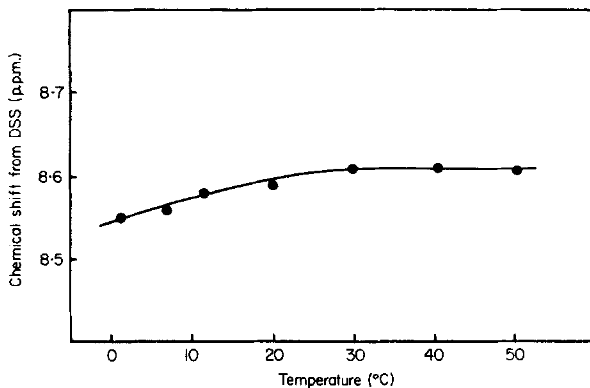


FIG. 7. Temperature dependence of the chemical shift of His12 in the S-peptide at pH 2·0, in 0·2 M-NaCl, ^2H_2O. Data generously provided by Dr A. A. Schreier. DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

In this pathway, two unfolded forms U_1 and U_2 are in slow equilibrium in unfolding conditions. Once refolding is initiated, the structural intermediates I_1 and I_2 are formed rapidly. The conversion of I_1 to I_2 is slow, and the native enzyme (N) is formed from I_2. As regards question (4), there is a standard explanation as to why α-helices might be formed at an early stage in folding. Helix formation is a co-operative reaction and several hydrogen bonds are formed. Even if each hydrogen bond has only a marginal stability, say 0·7 kcal per mole, forming six hydrogen bonds (as in the α-helix of residues 3 to 12 in RNAaseS) yields 4·2 kcal. Consequently, the helix can form without requiring stabilizing tertiary interactions from other parts of the molecule. Moreover, in model systems α-helices are formed in a time-range faster than microseconds (cf. Schwarz, 1965; Miller, 1973).

(c) *Folding intermediate or low-temperature form of unfolded RNAase A?*

The description of this species as a folding intermediate is based on the following considerations. (1) The change in chemical shift of one histidine residue (X) indicates that partial folding has occurred. The reasons for supposing that this is helix formation have been given above. Since a similar folding reaction takes place in the free S-peptide, it should be possible to test in other ways whether or not this is helix formation. (2) The change in chemical shift of X takes place only in refolding conditions. The data (not shown here) closely resemble those of the free S-peptide (Fig. 7). (3) The central question is whether the partial folding is a side reaction or whether it plays a central role in determining the pathway and increasing the rate of the subsequent folding process. The overall folding reaction studied here is the $U_1 \rightleftharpoons N$ reaction, in which the rate-limiting step is known to be an interconversion between two unfolded species ($U_1 \rightleftharpoons U_2$). Since this reaction can take place in conditions of complete unfolding (e.g. in 6 M-Gu·HCl, see Nall *et al.*, 1978), it may seem unlikely that partial folding should affect the rate of this interconversion reaction. In fact, it does: the rate of interconversion is approximately tenfold faster when preceded by partial folding, in refolding conditions (Nall *et al.*, 1978).

Two other considerations might be used to argue for describing this as a low-temperature form of unfolded RNAase A rather than as a folding intermediate. (1) The species is largely unfolded: three out of four histidine residues remain in unfolded environments. This is certainly correct, and it is fair to ask for other proofs of the functional role of the partial folding reaction before accepting it as an important step in folding. (2) The partial folding reaction is too fast to be measured in these experiments. This is not a valid objection to describing this species as a folding intermediate: the complete folding process of the fast-refolding species U_2 is also too fast to measure in these experiments.

(d) *Comparison with other studies of RNAase A folding intermediates*

The equilibrium transition curves for unfolding have been measured for the C-2 protons of the four histidine residues at pH 1·3 (Westmoreland & Matthews, 1973) and at pH 4·0 (Matthews & Westmoreland, 1973). At pH 4·0 only a single transition curve is seen and there is no evidence for an equilibrium folding intermediate. At pH 1·3 the unfolding transition curve of His12 precedes by 1 deg. C the common transition curve for the other three histidine residues, which suggests that at this low pH the S-peptide region of RNAase A can unfold first. In the Gu·HCl-induced and urea-induced unfolding of RNAase A (cf. Benz & Roberts, 1975), the unfolding transition

curves of individual protons are not reported but spectra suggest that His12 may also in these cases be involved in a local unfolding reaction that precedes the overall unfolding.

Stopped-flow studies of RNAase A unfolding show, in addition to the U_1 and U_2 forms of unfolded RNAase A mentioned above, a transient intermediate that is produced rapidly when native RNAase A unfolds at temperatures in and above the unfolding transition zone at pH 3·0 (Hagerman & Baldwin, 1976). This intermediate has not yet been characterized.

Calorimetric studies of the equilibrium unfolding transition (Tsong *et al.*, 1970; Tiktopulo & Privalov, 1974) show that there is a "pre-transition" zone in which a substantial heat uptake occurs at temperatures below the zone where co-operative unfolding occurs. Temperature-jump studies of unfolding show measurable kinetic processes only in the co-operative unfolding zone, when the absorbance change of the buried tyrosine groups is monitored (Tsong *et al.*, 1971). Thus, there is no evidence at present for unfolding reactions in the pre-transition zone that are linked to the co-operative unfolding transition. However, it is still possible that other probes of unfolding may reveal such a linkage. According to the analysis reported by Tiktopulo & Privalov (1974), the calorimetric criterion for the absence of any detectable intermediates (equality of the van't Hoff and calorimetric enthalpy changes) is nearly satisfied inside the transition zone for co-operative unfolding.

Measurements of attack by proteolytic enzymes have been used to search for intermediates in unfolding (Burgess *et al.*, 1975) and to postulate a structural pathway of unfolding (Burgess & Scheraga, 1975).

Laser Raman spectra have been used to measure the equilibrium unfolding transitions of various chromophores when RNAase A is unfolded by heating at pH 5. The results suggest that differences between the transition curves of different chromophores may be detectable by this technique (Chen & Lord, 1976).

We are particularly indebted to Dr C. R. Matthews for many suggestions concerning these experiments and for aid in performing them. Dr A. A. Schreier kindly provided some data, and we are grateful both to him and to Dr G. H. Snyder for discussion. Carol Cox participated in the preparation of samples and in calculating the results, and Virginia MacCosham chromatographed and assayed the protein samples. We appreciate their careful work. This research has been supported by grants from the United States National Science Foundation (BMS75-23510) and National Institutes of Health (GM19,988-16). One author (A. D. B.) is the recipient of a Public Health Services postdoctoral fellowship (1F22CA01960). We gratefully acknowledge the use of the Stanford Magnetic Resonance Facility (supported by NSF grant GR23633 and NIH grant RR00711).

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