Folding studies on ribonuclease A, a model protein
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Ribonuclease A (RNase A), an unusually well defined enzyme, has been a test protein in the study of a wide variety of chemical and physical methods of protein chemistry. These methods have in turn provided many insights into the functional properties of RNase A, as well as topics of general interest in protein biochemistry. The presence of four disulfide bonds and the existence of two cis peptide bonds preceding prolines in the native state have complicated the analysis of the folding pathway of RNase A. In this review, we present some new information about the folding of RNase A obtained recently by quench-flow H/D exchange combined with NMR and single-jump and double-jump stopped-flow techniques.

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Introduction
Protein folding, or how the amino acid sequence determines the three-dimensional structure of a protein, remains a major unsolved problem. The solution is not an easy one, as it requires the determination at the residue level of the interactions needed to account for the structures of the initial unfolded and the final native states, as well as those of all the intermediates and the transition states encountered in the folding reaction. Intermediates in the folding pathway are usually transient species, so a complete characterization of the folding pathway will require equilibrium structural analysis together with kinetic information [1].

In recent years, important progress has been made in the description of folding pathways mainly on the basis of new experimental approaches, such as multiple-jump stopped-flow techniques with detection by UV absorbance, CD or fluorescence; quench-flow of NH/ND exchange combined with two-dimensional NMR analysis; and the general use of site-directed single mutants and, in this context, the ‘protein engineering’ method [2] used to spot interactions in intermediates and transition states. These methods have been applied to a few model proteins and precise information has been obtained about their folding pathways (for reviews, see [3–8]).

Bovine pancreatic ribonuclease A (RNase A), an unusually well defined enzyme, has been a test protein in the study of a wide variety of chemical and physical methods of protein chemistry. These methods have in turn provided many insights into the functional properties of RNase A, as well as topics of general interest in protein biochemistry. Within the protein folding context, RNase A was the test protein for Anfinsen’s landmark experiment [9]. More recently, it has been used to implement the method of quench-flow combined with NMR-hydrogen exchange [10,11] to characterize structurally folding intermediates. However, a number of properties of RNase A, such as the presence of four disulfide bonds and the existence of two cis peptide bonds preceding prolines in the native state, have complicated the analysis of its folding pathway. We also have to add that, until recently, the expression of wild-type or mutant RNase A in sufficient yield was not possible [12].

Much of what we know about the folding intermediates of RNase A comes from the long-standing work of the groups of Baldwin and Scheraga. Baldwin and colleagues have pioneered work on trapping folding intermediates in RNase A and settled the basis for the quench-flow H/D exchange experiments. In addition to their early search for chain folding initiation sites, Scheraga and colleagues have fully exploited in recent years the double-jump stopped-flow technique and have used site-directed single mutants of RNase A in order to characterize the species present in the heterogeneous unfolded state caused by different X–Pro cis–trans conformations. As a consequence, important advances have been made in the detection of new kinetic phases and in the description of global features of the intermediates that transiently populate the refolding pathway. A detailed structural characterization of these intermediates as well as that of the transition state is still lacking and remains a challenge for future research.

In this review, we will first briefly describe the native state and what we know about the unfolded state both of the fully reduced and of the disulfide-intact protein. After a comment on the existing studies on oxidative refolding, we will focus on the folding and unfolding pathways of disulfide-intact RNase A as studied by the H/D exchange method and double-jump stopped-flow techniques.
The native state
RNase A is a single domain protein which contains 124 residues, with four native disulfide bonds at positions 26–84, 40–95, 58–110, and 65–72. The 3D structure of the native state has been determined in the crystal state [13] and in aqueous solution [14] (Fig. 1). It consists of three α-helices, helix I (residues 3–13), helix II (residues 24–34) and helix III (50–60), and an antiparallel β-sheet comprising β-strand I (43–49), β-strand II (61–63), β-strand III (72–74), β-strand IV (79–87), β-strand V (96–111), and β-strand VI (116–124). There are four proline residues: Pro42, Pro93, Pro114, and Pro117. In the native state, the peptide bonds preceding Pro93 and Pro114 adopt the cis conformation.

The entire β-sheet (with the exception of bulges) together with helix III are the most stable segments of RNase A as deduced from equilibrium H/D exchange measurements [14,15]. The four-stranded region of the β-sheet shown in Figure 2 has the highest density of the slowest exchanging amide protons. Helix III lies on top of this sheet with residues Val57–Cys58 in close contact with Tyr73–Ser75 and Ile106–Val108, thus configuring a hydrophobic core of exceptional stability against unfolding. The three-stranded part of the β-sheet, which is a prolongation to the right (Fig. 3) of the sheet region previously described, has its most slowly exchanging protons in the segment Phe46–His48, Met79–Thr82 and Ala102–Ile106 and in the site of attachment of the S-peptide segment (residues 1–20) mediated by the hydrogen bondings of His12(CO) and Asp14(NH) to the -NH and -CO of Val47, respectively. Helix II is connected to this part of the β-sheet via the disulfide bond between cysteines 26 and 84. The maximum conformational stability of RNase A is about 9 kcal mol⁻¹ and occurs between pH 7 and 9 at 25°C [16].

The unfolded state

Peptide fragments
Examination of nonrandom structures adopted by peptide fragments of a protein in isolation is now a well established method to get insights into the early stages of the folding process [17]. In essence, these studies try to establish the importance of local versus long-range interactions. A great number of RNase A fragments have been subjected to such studies with the aim of determining the conformation adopted when isolated and then evaluating the extent of their correspondence with native structural elements.

The N-terminal fragments 1–19 (or 1–20; S-peptide) or 1–13 (C-peptide) are the most studied RNase A fragments, probably because, following an initial study of its conformational properties by Brown and Klee [18], it was the first short linear peptide shown to form significant populations of an α-helix when isolated [19,20]. The observation contradicted the negligible helical populations predicted for such short peptides by helix-coil models [21,22] using values for the initiation, σ, and elongation, s, parameters determined in host–guest experiments [23]. The helix formed by either the C-peptide or the S-peptide was shown to stop at Met13 [24] and span the same residues as in the intact protein [20]. The α-helical population, which is in dynamic equilibrium with the unfolded conformation, decreases with increasing temperature, indicating that helix formation is an enthalpy-driven process [19]. The equilibrium is also affected by pH and TFE concentration. The pH dependence of the helix population, a bell-shaped curve with two inflexion points corresponding to two pKₐ's (4 and 7), led to the proposal of two pH-dependent sidechain interactions, a salt-bridge Glu2(COO⁻)…Arg10(Gdm⁺) [25,26] and a pseudo-stacking interaction between the aromatic rings of Phe8 and
His12 [27]. According to results of quantum-chemical calculations [28], His12 interacts more strongly with Phe8 than does His12. These two short-range (or medium-range) interactions able to direct the formation of the \( \alpha \)-helix in the peptide are also present in the intact protein. Interactions between the helix macrodipole and charged groups near the ends of the helix may either stabilize or destabilize the helix [29]. Recently, theoretical simulations that consider only the free energy of local mainchain electrostatic backbone, mainchain hydrogen bonds and the burial of nonpolar area have defined the Glu2–Met13 region of RNase A as an independent folding unit [30].

Peptide segments involving helix II and helix III have also been analyzed by NMR methods [31,32]. Proton chemical shift variation with temperature and addition of stabilizing (TFE) or denaturing (urea) agents provided experimental evidence for the existence of a small but significant population of an \( \alpha \)-helix spanning residues Leu51–Cys58 in agreement with that found in the intact protein. The helical population is enhanced in 30% TFE and by lowering the temperature to 0°C. The isolated Ser21–Pro42 fragment is also able to adopt a measurable population (14% at 22°C, pH 5.4) of a native-like \( \alpha \)-helical structure. The formed helix spans the same residues as those that are helical in the native protein (Asn24–Asn34). The finding that all three helices of RNase A are partially stable as short fragments in the absence of tertiary interactions provides experimental support for the idea that \( \alpha \)-helices act as chain folding initiation sites.

By computing the free energy of hydrophobic interactions leading to short hairpin-like conformations, Scheraga and co-workers [33] identified several chain folding initiation sites in RNase A, the most stable of which was determined to consist of residues 106–118 (Fig. 2). The fragment 105–124 was labeled with appropriate acceptor and donor fluorescent probes at the N and C terminals, respectively, and examined by nonradiative energy transfer [34]. Energy transfer was monitored using both fluorescence intensity (sensitized emission) and lifetime (donor quenching) experiments. The lifetime data indicate that the peptide system is a dynamic flexible one. A detailed analysis of the data showed a relatively narrow distribution of interprobe distances and hence the existence of a partially ordered structure. Changes of the width and position of the average end-to-end distance distributions with temperature and addition of 6 M GdnHCl suggested that the nonrandom structure is stabilized by hydrophobic interactions and that in this case the addition of denaturant unfolds the peptide, in contradiction to that observed on the unlabeled peptide.

Rico and co-workers examined the conformational properties of the C-terminal 112–124 fragment of RNase A by CD and \( ^1H \) and \( ^13C \)-NMR spectroscopy [35]. Only sequential \( \alpha N(i,i+1) \) and intraresidue NOE crosspeaks were observed in the NOESY spectra, a fact that points towards an essentially extended polypeptide chain. No signs of the native Gly112–Tyr115 \( \beta \)-turn were detected, which suggests that long-range interactions may be needed to stabilize this particular \( \beta \)-hairpin segment, as has been concluded recently by Scheraga and co-workers [36] from a time-resolved fluorescence study of the entire polypeptide chain.

A short peptide containing residues 58–72 of RNase A [37] was shown to form the native disulfide bond between cysteines 65 and 72 about four times more favorably than the nonnative one between 58 and 65. Scheraga and coworkers also examined the folding properties of several fragments in the region Ser50–Met79 [38,39], where three cysteines, Cys58, Cys65 and Cys72, are present. The latter two form a disulfide bridge in the native structure with a type III \( \beta \)-turn between residues Cys65 and Gly68. By incubation under oxidizing conditions of the free thiol peptide, it was established that the formation of the native disulfide bond is thermodynamically preferred over the formation of the nonnative bond, 58–65. This is so even though the entropy of ring closure in both cases would be expected to be similar because they give rise to rings of the same size. NMR analysis of the peptide 61–74 [38] shows that short-range interactions help to stabilize the Cys65–Cys72 disulfide bond with the formation of a type-II \( \beta \)-turn involving
residues 66–69, a turn that is shifted by one residue from the native type III turn at residues 65–68.

In summary, sound evidence from the analysis of RNase A fragments points to short-range interactions being operative in the formation of low but significant populations of native structural elements. It is reasonable to expect that the intrinsic stabilization of the structures present in these segments will operate also in the unfolded state of the entire polypeptide chain under native conditions.

The intact polypeptide chain
Time-resolved fluorescence measurements have been used [36] to determine the extent of nonradiative excitation energy transfer between donor and acceptor probes in RNase A so as to determine the extent of residual structure in denatured states of RNase A. Analysis of the decay curves allowed the determination of parameters describing the distribution of interprobe distances and the diffusion coefficients of the ends of the segments defined by the probes. The fluorescent donor was set at the C terminus and the acceptor at the e-amino group of either Lys1, Lys61 or Lys104. Apart from the native state, three denatured states were tested: the disulfide-intact unfolded state (U), the reduced state under native conditions (R_N), and the reduced state at high denaturant concentration (R). The determined mean values for end-to-end distance distributions for (61–124) RNase and (104–124) RNase in the partially denatured states (U and R_N) as well as for the totally unfolded state (R) are lower than those calculated for statistical random coil states of peptides with the same probe separation. This means that even in the fully denatured state there exist some residual folded structures. All the determined parameters (transfer efficiencies, mean values of interprobe distributions, their widths as well as diffusion coefficients) point towards substantial refolding accompanying the transitions from R to R_N or U. The end-to-end distance and thus the structure formed in the C-terminal segment even in the unfolded state does not correspond to the native structure (an antiparallel β-sheet). By comparing with the results obtained for the isolated peptide, it was concluded that the structure formed is stabilized by both local and nonlocal interactions.

The thermally denatured state of RNase A has been examined by small-angle X-ray scattering and Fourier transform infrared spectroscopy and found to be on average a compact structure having residual secondary structure [40]. Under strongly reducing conditions, the protein further unfolds into a looser structure but still retains a comparable amount of secondary structure. The dimensions of the thermally and chemically denatured states of the reduced protein are different but are more compact than is predicted for a random coil of the same length.

Indirect evidence for residual structure in the disulfide-intact unfolded state of RNase A comes from H/D exchange experiments [14,15]. It is well known that isotopic exchange of the slowest exchanging protons in a protein can be used as indicators of global stability [41].
Exchange of amide protons in proteins normally belongs to the EX2 limit, in which the refolding rate constant $k_r$ is much larger than the intrinsic exchange rate, $k_c$. Under these conditions, the observed exchange rate is the product of the latter ($k_r$) and the equilibrium constant of the opening reaction. The free energy of RNase A unfolding as deduced from exchange is much larger than the calorimetrically determined thermodynamic value. The most plausible explanation of this discrepancy is that the intrinsic $k_c$ values, corresponding to a structureless polypeptide, are no longer valid for the unfolded state of RNase A under native conditions. Consequently, agreement with experiment requires that the configurations present in the unfolded state of RNase A under native conditions must have strongly retarded rates (up to three orders of magnitude) relative to the intrinsic exchange rates of model peptides. They must be partially folded structures or at least more closed forms than fully denatured conformations.

**Oxidative refolding and reductive unfolding of RNase A**

The study of protein folding through disulfide bond formation involves the thermodynamic, kinetic and structural characterization of all possible disulfide intermediates. Assuming that conformational folding processes are coupled to the formation of disulfide bonds, the analysis of the disulfide intermediates should provide insights into the folding pathway. Since in RNase A the rate-limiting step of the folding reaction is coupled to the conformational folding process and not to disulfide bond rearrangement [42], RNase A is actually a good model to study folding through disulfide bond formation.

Starting from a fully reduced RNase A, the products of reoxidation in the presence of 8 M urea, after being trapped by quenching any remaining free thiol group, were shown to be a mixture of multiple molecular species (the so-called ‘scramble’ RNase A). If we concentrate on the one-disulfide bond intermediates, which are the ones best characterized, the work of Anfinsen and Harber [43] indicated the existence of a random distribution. However, recent experiments by Ruoppolo and co-workers [44], using similar refolding conditions to those employed in [43] followed by enzymatic cleavage, separation and identification of fragments by mass spectrometry, have shown the existence of only 14 out of 28 possible one-disulfide species. Interestingly, all the native disulfide bonds were detected with the exception of the one between 26 and 84. Although 14 is a significant fraction of the theoretical total, the occurrence of only a subset of disulfides clearly suggested that the formation of the S-S bridges does not occur at random, even when reoxidation takes place under denaturing conditions. When the reoxidation was performed under nondenaturing conditions, the formation of several well defined nonnative as well as native S-S bonds was observed at early stages of the folding process. At later stages of refolding, nonnative disulfides were greatly diminished or absent.

By using the redox pair DTT$^{\text{red}}$/DTT$^{\text{ox}}$ and a novel thiol-blocking reagent, (2-aminoethyl)methanethiosulfonate (AEMTS), Scheraga and co-workers [45,46] have studied the regeneration of RNase A from the reduced to the native form. The one-, two-, and three-disulfide intermediates achieve a steady-state distribution after which the native protein regenerates. None of these disulfide-containing intermediates has any significant enzymatic activity, thus demonstrating that these intermediates are considerably disordered. The one-disulfide intermediates were the subject of a more quantitative analysis [46], in which it was shown that 24 out the 28 theoretically possible intermediates were present in significant populations, the distribution of which is certainly nonrandom. All of the four native disulfide pairings have populations greater than those predicted by loop entropy calculations, suggesting the presence of enthalpic contributions stabilizing these species. The species containing the disulfide bond between cysteines 65 and 72 comprises 40% of the entire one-disulfide population. The observation of a steady-state distribution of one-disulfide intermediates as well as of the two- and three-disulfide species indicates that the distribution of species is thermodynamically controlled. Then it appears likely that the 65–72 disulfide bond will be highly populated in the latter stages of folding, and this should favor regeneration pathways in which that bond forms first.

According to Scheraga and co-workers [47], the reductive unfolding of RNase A proceeds through parallel pathways with the formation of two well populated partially unfolded three-disulfide intermediates, des[65–72] and des[40–95]. Two distinct local unfolding events, rather than a global one, are involved in the rate-limiting step, contrary to the view of an all-or-none mechanism. Both species des[65–72] and des[40–95] have also been observed to be populated during the oxidative refolding of RNase A with DTT. On that basis, the authors suggest that the oxidative refolding of RNase A from the fully reduced state proceeds through two consequentially different transition states. The stability and solution structure of des[65–72] with the free thiol blocked by AEMTS was determined by examining its thermal transition curve and NMR spectroscopy [48]. The disruption of the 65–72 disulfide bond decreases the thermal stability of the protein by ~5 kcal mol$^{-1}$, but the structure of the modified protein is closely similar to RNase A in all regions except for the loop region comprising regions 60–72. The conclusion is that, in reduction pathways that include des[65–72] RNase A, the rate-determining step corresponds to a partial unfolding event in one region of the protein and not to a global conformational unfolding process.
NMR and quench-flow labeling studies on the RNase A folding and unfolding pathway

The folding pathway

The introduction of pulsed quench-flow H/D exchange methods combined with 2D NMR spectroscopy opened a new and efficient way to obtain highly detailed structural information about the folding reaction by allowing the characterization of structurally transient folding intermediates (for recent reviews, see [4,5,49]). The method is based on the fact that protein amide hydrogen atoms not involved in hydrogen bonds exchange rapidly with solvent. The experiment is as follows. The protein is initially unfolded in denaturing conditions in D₂O, so that all the amide protons are deuterated. In a first mixing step, the solution is diluted to initiate folding and a time is allowed for the protein to refold before a labeling pulse in the form of a sharp pH increase is applied. Amide sites still exposed at this stage are selectively protonated. After the pulse, exchange is quenched by lowering temperature and pH down to minimal exchange values. Then, the protein is allowed to refold to the native form and the labeled amide sites assayed by NMR. Proton occupancies measured as a function of the refolding time are used to determine the exchange rate constants and from them the protection factors of individual amide sites, P ≡ kₑ/kₑₓ, are evaluated. The protection factors indicate whether a given proton is hydrogen bonded and, consequently, belongs to a secondary structure element or a tertiary interaction. The appearance of protection as a function of time informs us about the order in which the different elements are formed in the refolding.

RNase A has played a major role in the development of the quench-flow methodology and important information about the intermediates in its folding pathway has been obtained. ¹H–³H exchange labeling and optical studies performed by Baldwin and colleagues [50,51] early on allowed them to make a proposal for the pathways of unfolding and folding of RNase A:

<table>
<thead>
<tr>
<th>Folding pathways:</th>
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<tbody>
<tr>
<td>~20%</td>
<td>Uᵢ → N</td>
</tr>
<tr>
<td>~15%</td>
<td>Uₛ I → N</td>
</tr>
<tr>
<td>~65%</td>
<td>Uₛ II → I₁ → Iₛ → N</td>
</tr>
</tbody>
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<table>
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<tr>
<th>Unfolding pathways:</th>
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<tbody>
<tr>
<td>N → Uᵢ</td>
<td></td>
</tr>
<tr>
<td>Uᵢ → Uₛ I</td>
<td></td>
</tr>
<tr>
<td>Uᵢ → Uₛ II</td>
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</tbody>
</table>

Unfolded RNase A (U) is kinetically heterogeneous, being a mixture of a fast-folding species, Uᵢ (~20%), and at least two slow-folding species, Uₛ I (~15%) and Uₛ II (~65%), each of which folds to native RNase A (N) by an independent pathway. The scheme for the refolding of Uₛ II is the simplest possible minimal model that accounts for the experimental data [10,11]. First, a hydrogen-bonded intermediate is formed, I₁, followed by a native-like intermediate, Iₛ, which finally refolds to the native protein. The unfolded forms Uᵢ, Uₛ I and Uₛ II most probably differ in the cis–trans configuration of Pro93 and Pro114, both of which are cis in the native protein.

The intermediate I₁ had been detected in early studies of RNase A folding intermediates [50,51] but had not been characterized structurally. Formation of N (from Uᵢ) and Iₛ (from Uₛ II) can be monitored in optical stopped-flow experiments by the absorbance change that accompanies burial of tyrosine sidechains. A decay curve of proton occupancy as a function of the refolding time can be constructed on that basis, which should correspond to the kinetic curve for the formation of Iₛ + N (see dashed line in Fig. 2 in [11]). The sum Iₛ + N falls to 80% near t = 0 as Uᵢ folds and has the value of 15% at 4s, after Uₛ II has folded, because Uₛ I has only begun to fold. On this basis, the early presence of an individual amide proton with substantially greater protection than that predicted by the decay curve would indicate that a folding intermediate (I₁) is populated and that this amide proton forms part of its structure. Those probes that show an exchange behavior superimposable with the Iₛ optical decay curve are part of a structure that is stabilized only when Iₛ is formed. A number of protons belonging to the β-sheet and helix III of RNase A show much larger protection factors than the one corresponding to the formation of Iₛ. On the other hand, three probes are found unprotected in I₁: His12 and Met13 in helix I, and Glu49 in the main β-sheet. The intermediate I₁ is evidently highly stable and contains a large part of the secondary structure: practically the entire β-sheet and helix III and probably helix II. Only helix I appears to be stabilized later in the step I₁→Iₛ. The intermediate I₁ is formed within the first 100 ms. Two major disagreements were found with the results expected for a sequential model of the folding pathway. First, I₁ is not fully populated in the Uₛ II→I₁ step. Second, the proton-exchange properties of I₁ are not constant along the refolding time. There is no definite explanation for the first observation. In the second case, it can be considered that I₁ actually contains a broad distribution of species as regards the protection factors of the probes in I₁ and that this distribution changes gradually with time. In other words, the β-sheet is formed rapidly and cooperatively but is only marginally stable when it is first formed. Subsequent sidechain interactions stabilize it.

In a later work, Udgaonkar and Baldwin [52] carried out a further characterization of the nature of the early folding
intermediate of RNase A. They concluded that I₄ is not a typical molten globule intermediate, but rather has some fixed sidechain structure. This conclusion was reached from the ability of I₄ to bind 2′-CMP as deduced from stopped-flow experiments monitored by the change in absorbance of 2′-CMP at 254 nm that accompanies its binding to RNase A [53,54]. Analysis of the exchange properties of the protected amide protons probes reveals that both the number and the extension of the protection is similar in I₄ and I₅, thus confirming that I₄ is highly structured and leaving as the major difference between the two intermediates the fact that the sidechains of Tyr25, Tyr73, Tyr92 and Tyr97 are buried in I₅ but not in I₄.

The unfolding pathway

Baldwin and his group have also addressed the problem of the unfolding pathway of RNase A. Previous experiments based on optical probes had shown that secondary and tertiary structures unfold with the same kinetics, thus suggesting that there are no unfolding intermediates [55]. In their most recent experiments [56], the approach used was that of working under conditions (10°C, pH 8.0, 4.5 M GdnHCl) where the refolding rate is much lower than the rate of intrinsic chemical exchange of unprotected protons, i.e. under conditions of the EX₁ exchange mechanism [57]. In this way, they were able to measure by quench methods individual exchange rates which are equivalent in these conditions to unfolding rates. The 43 slowest-exchanging protons undergo exchange exclusively by the EX₁ mechanism and show closely similar rates. On the other hand, the apparent unfolding rates measured by CD and hydrogen exchange are different. This could in principle be explained by the presence of an intermediate with distinct properties. However, the data from the two techniques can be made consistent by taking into account the effect of proline isomerization after unfolding as well as the different dependence of the apparent unfolding rate, kₐpₐp, in the H/D exchange method (kₐpₐp = kₐₚ) and in CD (kₐpₐp = kₐ + kₚ). These joint results indicate that a single rate-limiting step in unfolding breaks the entire network of peptide hydrogen bonds and causes the overall unfolding of RNase A.

In a later report, however, Baldwin and co-workers [58] claimed to have detected the first reported intermediate in the unfolding pathway. The evidence is based on the abrupt reduction in the intensity of upfield shifted resonances (a methyl group of Val63 and a CβH proton of Pro117) immediately after introduction of the denaturing conditions. After this initial drop, the kinetics of disappearance of the lines of the native protein follows the kinetics measured by CD. The results can be explained by a simple kinetic model for unfolding:

$$N \xrightarrow{\text{fast}} I \xrightarrow{\text{slow}} U$$  Model 1

in which the intermediate is rapidly formed from the native state but exchanges slowly (> a few ms) with N in the NMR timescale. The intermediate resembles the native structure with regard to the secondary structure and amide exchange, but with the sidechains free to rotate so as to be able to average the effect of aromatic ring currents on shifts. The authors conclude that the intermediate appears to be a “dry molten globule” of the kind hypothesized by Shakhnovich and Finkelstein [59], with the rate-limiting step for unfolding occurring later, when water enters the hydrophobic core and the hydrogen bond network is broken.

Folding of RNase A by double-jump stopped flow

Assigning the different kinetic species present in the unfolded state of RNase A

The exact nature of the different unfolded species of RNase A in strongly native conditions, Uₕ, Uₕ, I, and Uₕ II, was not unambiguously elucidated at that stage. Cis–trans isomerization of proline residues after unfolding was suggested to be the origin of the heterogeneity in the unfolded forms [60]. This was confirmed experimentally by site-directed mutagenesis of Pro93 and Pro114 which are cis in the native structure [61], although a more complete characterization was not possible due to the unexpectedly complex kinetics shown by these mutants.

Recently, Scheraga and co-workers [62] have investigated the refolding and unfolding of disulfide-intact RNase A by using single-jump and double-jump stopped-flow techniques. Double-jump experiments consist essentially of two steps or jumps: in the first one, the folded protein is unfolded at high GdnHCl concentration and low pH for a delay time long enough to populate the species with all native X-Pro peptide bonds, but short enough not to form any of the resulting species from cis–trans or trans–cis isomerization. In the second step, the purely conformational refolding of the first species can be studied in a variety of conditions. Conditions (1.5 M GdnHCl and low pHs and temperatures) were chosen so that the folding process is slowed down and the refolding phase corresponding to Uₕ occurs on a timescale of seconds, in contrast to more native conditions where the time constant is on the order of milliseconds. Under these slow folding conditions, a new very fast phase was observed with a time constant of 50 ms and an amplitude of ~6%, in addition to the usual fast and slow phases that involve the unfolded species Uₕ and Uₘ, respectively. Under more favorable folding conditions (0.58 M GdnHCl, pH 5.0 and 15°C), the presence of a fifth unfolded species was detected. It has a refolding time constant on the order of 2 s under the conditions employed and was labeled Uₘ, for medium-refolding species. Single-jump refolding experiments monitored by tyrosine burial and by 2′-CMP inhibitor binding indicate that the five unfolded species refold to the native state along independent refolding pathways [63].
The authors also propose that the medium-folding species of wild-type RNase A consists of two unfolded species. One has only the Tyr92→Pro93 in a nonnative (trans) state, an assignment that is based on the similarities of the kinetic constant of the first species to appear in Pro93→Ala with the Uf species in wild-type RNase A. The other one has only the Tyr92→Pro93 peptide bond in the native state since, in the double-jump experiments on Pro114→Ala RNase A (no significant population with the Asn113→Pro114 in a cis state), a Uf species is observed. The remaining three species, all of them with a Tyr92→Pro93 nonnative trans peptide bond, must belong to the Uf species. Unfortunately, it was not possible to distinguish between conformers belonging to species Uf I and Uf II. The proposed model for the unfolding of RNase A first by a pure conformational step to form Uf, the species with all X-Pro bonds in the native conformation, and thereafter by successive and independent cis-trans and trans-cis isomerization events at the peptide bonds of X–Pro93, X–Pro114, and X–Pro117 is shown in Figure 4.

Intermediates in the folding of the species with all X-Pro bonds in the native conformation

Once established the delay time (0.9 s at 5°C) between jumps to populate Uf at ~100% without being complicated by the presence of nonnative X-Pro peptide bonds [62], the effects of pH, temperature and GdnHCl concentration on the refolding rates, kvf, were examined [66]. When studying the dependence on pH, the unexpected result that the reaction rates decreased when the pH raised from pH 2 to 6 was found. Also, the changes in the reaction rates

\[ k_{cis} > k_{trans} \]

The rationale behind the assignment of cis-trans isomers to the kinetically detected species is that the population of the cis configuration in the different Pro→Ala mutants in the unfolded state is negligible compared to the one in the wild type. Thus the mutant Pro93→Ala does not show in the refolding process a Uf phase, so that the Uf species must have a cis-Pro93 configuration. Among the species complying with that condition, the one with all the proline isomers in the native state except that between Asn113 and Pro114 should be assigned to Uf because the folding rate constant and relative amplitude of the fast-folding species of the mutant Pro114→Ala satisfactorily coincide with those of the Uf species of wild-type RNase A. On the other hand, the observed fact that the isomeric state of the Val116→Pro117 bond affects only the slow-folding species indicates indirectly that the isomer with all X-Pro bonds in the native state except the one between Val116 and Pro117 belongs also to the fast-folding species, Uf.
were sensitive to two pK_s, corresponding to a carboxylic acid and a histidine. Furthermore, the variation in the rate constants was much greater at the high GdnHCl concentration than at the low one. This cannot be explained in terms of a simple two-state transition. The observed experimental data point to the presence of an intermediate, I_u, with some unusual characteristics. Further support for the presence of the intermediate comes from the nonlinearity of the GdnHCl-dependence data of lnK_{vf}. Hence, the following sequential model was proposed:

\[
U_{vf} \rightarrow I_u \rightarrow I_\phi \rightarrow N \quad \text{Model 2}
\]

It is assumed that, when the unfolded state U_{vf} is placed under folding conditions, it undergoes a rapid pre-equilibrium to form the intermediate. This intermediate then proceeds to the native state passing through the rate-limiting step. The observed pH shifts, the extent of burial of the solvent-exposed surface area as a function of temperature, and the determined kinetic and thermodynamic parameters are all consistent with the fact that the intermediate is formed by a hydrophobic collapse. The intermediate presents properties similar to those of equilibrium molten globules. I_u is highly populated at low pH and low denaturant concentrations, whereas at higher pH and higher denaturant concentrations, the population of the intermediate decreases and the folding reaction approximates a two-state process. The experimental data also suggest that the rate-limiting transition state in the refolding pathway results from the formation of ordered structure within the hydrophobically collapsed intermediate. On the basis of changes in the buried surface area between the intermediate and the transition state, the authors conclude that the transition state in the refolding pathway is not native-like and is not identical to the one on the unfolding pathway. However, some of these conclusions have to be considered with caution since they are based on changes in parameters whose magnitudes are close to the experimental errors.

By using stopped-flow CD at 222 and 275 nm under the same conditions employed in the previous absorbance or fluorescence detected experiments, Scheraga and colleagues have shown the presence of a burst phase (i.e. a change in the CD signal corresponding to the unfolded state within the dead time of the mixing device) in the refolding of U_{vf} [67]. This burst phase was not detected by absorbance nor by fluorescence under any conditions explored. The change in CD in the burst phase suggests the presence of an early new intermediate, I_u, before I_\phi is formed. At the initiation of folding, U_{vf} is converted to a largely unfolded intermediate, I_u, which then undergoes a hydrophobic collapse to form the molten globule like intermediate I_\phi, according to:

\[
U_{vf} \rightarrow I_u \rightarrow I_\phi \rightarrow N \quad \text{Model 3}
\]

The CD values obtained for I_u and I_\phi indicate that I_u has no secondary structure and presumably differs from U_{vf} by a local structural rearrangement, while I_\phi has a substantial population of secondary and tertiary structures, about 40–50% of that of native. The structures of I_u and I_\phi have been addressed by NMR quench-flow experiments and theoretical kinetic simulations [68]. I_u was found not to have any stable secondary structure. On the contrary, in I_\phi, a large part of the β-sheet as well as helix II appear to be already formed while the rest of the protein molecule remains unstructured. The protection factors in I_\phi are low, indicating that this intermediate has a dynamic structure consistent with I_\phi being a molten globule like intermediate. The regular structure formed in I_\phi is much less than that observed in the intermediate I_1 detected previously in the refolding of the slow species U_S II [10,11,52]. The stability of I_\phi is also lower than that of I_1. Therefore, a slower refolding rate results in the formation of more stable intermediates.

Very recently, Scheraga and co-workers prepared the mutant Tyr92→Trp in order to obtain site-specific information about the folding of the protein by fluorescence-detected single-jump and double-jump stopped-flow techniques [69]. The hydrophobically collapsed intermediate I_\phi was observed directly as a burst phase (quenching during the dead time of mixing) in the double-jump experiment, also implying that the region around Trp92 is involved in the formation of I_\phi.

**Conclusions**

In summary, NMR combined with quench-flow exchange techniques and single- and double-jump stopped-flow kinetic studies have provided us with added structural information about kinetic intermediates in the refolding of RNase A. Advances have been made also in our knowledge of the effect of cis-trans isomerizations of the different X-Pro bonds on the refolding rates. However, it can be seen that even for an extremely well known protein such as RNase A, the folding reaction is far from being understood. All available information, some of which appears to be contradictory, should be tested and unified.

Starting from the native state, there is sound evidence on the existence of different elements of secondary structure in significant populations. However, the first intermediate in the refolding of U_{vf} appears unstructured, showing only local structural rearrangements. In the refolding of the slow kinetic phase, U_S II, in strongly native conditions, the first detected intermediate, I_1, shows already an almost complete secondary structure. A more precise knowledge of the steps between the conditions characterizing the refolding protein and the formation of the secondary structure is urgently needed, but that point will certainly have to wait until the development of submillisecond time-resolved techniques. It would be also very
helpful to have a more detailed knowledge of the denatured state of RNase A, an objective that can be addressed at the present time by multidimensional analysis of the $^{15}$N- and $^{13}$C-labeled protein.

The structure, properties and stability of the intermediates found under different refolding conditions, either strongly native or slightly denaturant, present some important differences, like those found between $I_0$ and $I_1$, on which we have already commented. Whether these differences depend solely on the fact of the refolding being rapid or slow should be more thoroughly tested. Up to the present time, all refolding schemes for RNase A correspond to the simplest possible minimal model to account for experimental data. Also, in these models, the intermediates are placed in the productive way. The possibility that the intermediates are off-pathway, a point that has been raised recently [3], has not been considered because of the lack of sufficient experimental data to discriminate between the two mechanisms.

Information about the folding transition state is equally limited and even contradictory. Whereas according to the refolding of $U_g$ II, the transition state, which follows $I_0$ (a highly structured intermediate), should be native-like, the conclusions of Scheraga and colleagues from the refolding of $U_g$ are that the transition state is not native-like and is not identical to the one in the unfolding pathway. Differences are also found in the unfolding pathway. By monitoring unfolding by hydrogen exchange the conclusion was reached that all slowly exchanging protons behave uniformly, thus indicating that unfolding is a global process. In contradiction of this, the results of Scheraga and co-workers from the reductive unfolding of RNase A indicate the existence of two distinct local unfolding events involved in the rate-limiting step, rather than a global one. More work is needed in the particular context of the reductive unfolding of RNase A, such as the structural characterization of more trisulfide and disulfide species by NMR methods. In more general terms, the thermodynamic and kinetic analysis of the folding and unfolding reactions for selected RNase A mutants appears to be needed for a more complete characterization of folding intermediates and of the rate-limiting transition state.

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References