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Circular Dichroism, Raman Spectroscopy, and Gel Filtration of Trapped Folding Intermediates of Ribonuclease†

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ABSTRACT: The intermediates of ribonuclease with one to four disulfide bonds trapped during refolding of the reduced protein have been compared to the fully reduced and native forms of the protein by gel filtration, circular dichroism, and Raman spectroscopy. Correctly refolded ribonuclease is indistinguishable from native protein, while a three-disulfide intermediate has a compact conformation which is very similar, but not identical. In contrast, all other intermediates with one to four disulfide bonds are qualitatively similar to fully reduced ribonuclease by their circular dichroism and Raman spectra, although the disulfide cross-links affect the dimensions of the polypeptide chain. The apparent absence of stable partially ordered structures in the initial disulfide intermediates implies that during refolding there are relatively few constraints on formation on disulfide bonds, although their formation is probably not entirely random. The stable conformation appears during refolding only when the three or four disulfide bonds capable of stabilizing a native-like conformation of the entire polypeptide chain occur simultaneously.

Bovine pancreatic ribonuclease A is one of the most extensively studied proteins [reviewed most recently by Richards & Wyckoff (1971)] and remains the classic subject of experimental studies of protein unfolding and refolding. There is still no general consensus as to the mechanism or pathway of the refolding.

Many studies have been made of unfolding and refolding with the four disulfide bonds kept intact. Most equilibrium measurements of the reversible unfolding transition produced by denaturants, high temperatures or pressures, extremes of pH, or combinations of these factors have indicated that the transition is cooperative, with essentially only the fully folded and fully unfolded states populated at equilibrium, all partially

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folded states being thermodynamically unstable (Hermans & Scheraga, 1961; Ginsburg & Carroll, 1965; Brandts & Hunt, 1967; Brandts et al., 1970; Salahuddin & Tanford, 1970; Privalov & Khechinashvili, 1974; Benz & Roberts, 1975a,b; Nall & Baldwin, 1977). In addition, there are numerous indications of partial conformational changes in the folded state under conditions preceding the full unfolding transition (Rupley & Scheraga, 1963; Simons et al., 1969; Privalov et al., 1973; Tiktopulo & Privalov, 1974; Westmoreland & Matthews, 1973; Benz & Roberts, 1975a,b; Chen & Lord, 1976; Matheson & Scheraga, 1979b; Howarth, 1979). In contrast, other investigators claim that small but detectable concentrations of molecules with partially folded conformations are present through the transition region (Burgess et al., 1975; Burgess & Scheraga, 1975; Chavez & Scheraga, 1977; Matheson et al., 1977; Matheson & Scheraga, 1979a; Howarth, 1979; Chavez & Scheraga, 1980; Konishi & Scheraga, 1980).

Unstable intermediates are more likely to accumulate to detectable levels, although only transiently, during the kinetic time course of unfolding and refolding (Baldwin, 1975). However, the kinetic complexities observed with RNase, with the four disulfides kept intact, now appear to be due primarily to two or more slowly interconverted forms of the unfolded protein, one of which refolds rapidly (Garel & Baldwin, 1973, 1975; Garel et al., 1976; Hagerman & Baldwin, 1976; Nall et al., 1978). They probably arise by relatively slow cis–trans isomerization of the peptide bonds adjacent to proline residues (Brandts et al., 1975, 1977; Lin & Brandts, 1978; Schmid & Baldwin, 1978; Cook et al., 1979). This phenomenon greatly complicates experimental study of refolding not limited by proline cis–trans isomerization. It is also possible that the four residual disulfide cross-links in the unfolded state, which consequently is not fully unfolded, play an important role in determining the mechanism and pathway of refolding from that state.

Unfolding of RNase[1] after reduction of the four disulfide bonds, to produce a fully unfolded polypeptide chain, is also reversible (Sela et al., 1957). Although the refolding that accompanies disulfide formation in reduced RNase has been studied extensively (Anfinsen et al., 1961; White, 1961; Haber & Anfinsen, 1962; Epstein & Goldberger, 1964; Givol et al., 1964; Anfinsen, 1967, 1973; Hantgan et al., 1974; Ahmed et al., 1975; Schaffer et al., 1975; Schaffer, 1975; Chavez & Scheraga, 1977; Takahashi et al., 1977; Garel, 1978; Chavez & Scheraga, 1980; Konishi & Scheraga, 1980), there is no general consensus as to the mechanism of the process. One of us has recently examined the kinetics of disulfide bond formation and breakage in RNase and has isolated the kinetic intermediates which accumulate during refolding, trapping them in a stable form by blocking irreversibly the thiol groups of free cysteine residues by reaction with iodoacetate (Creighton, 1977, 1979). It was concluded that the folding pathway converges relatively slowly with increasing disulfide bond formation to a limited number of intermediates, although the pathway of disulfide formation is complex and is not yet known. Disulfide bond formation can serve as a probe of the conformational transitions occurring during refolding (Creighton, 1978), so it is of interest to determine the conformational basis of the nonrandom disulfide bond formation. One means of accomplishing this is by examination of the conformational properties of the trapped intermediates, as has been shown for bovine pancreatic trypsin inhibitor (Creighton et al., 1978; Kosen, 1978; Kosen et al., 1980), the folding pathway of which is known in detail (Creighton, 1978).

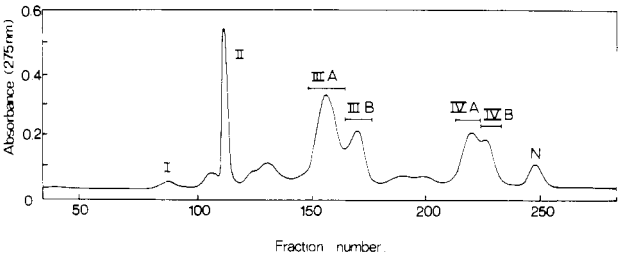

FIGURE 1: Chromatographic separation of disulfide intermediates trapped during the refolding of reduced RNase, illustrating the partial resolution of the peak containing primarily incorrect four-disulfide species into peaks IVA and IVB. Refolding was quenched by iodoacetate 20 min after initiation upon addition of oxidized glutathione to 0.2 mM. Chromatography on CM-cellulose and other manipulations were performed as described previously (Creighton, 1979). The elution profile was determined by continuous measurement of the absorbance at 275 nm. The resolution of the trapped species into the two-disulfide species II, three-disulfide peaks IIIA and IIIB, and four-disulfide peaks IVA and IVB, plus the correctly refolded N, is illustrated. Fully reduced and one-disulfide forms of the protein had disappeared after 20 min of refolding.

Here we report further studies of the trapped RNase intermediates, using gel filtration to fractionate the intermediates further and to measure their overall compactness, circular dichroism to look for regular secondary structure of the polypeptide backbone and asymmetric environments of the aromatic and disulfide chromosphores, and laser Raman spectroscopy to examine the conformation and environment of various parts of the polypeptide chain.

Methods

Proteins. Fully reduced RNase and folding intermediates were trapped at varying times during refolding of reduced RNase, with oxidized glutathione as disulfide reagent, by rapid alkylation of all free thiol groups by iodoacetate. The trapped intermediates were purified by column chromatography on CM-cellulose, as in Figure 1 of Creighton (1979). The chromatographic preparation of the nonnative four-disulfide intermediates used here gave a partial resolution into two peaks, designated IVA and IVB, greater than that observed previously, so the chromatographic profile is shown in Figure 1. The various forms of RNase eluted from the column were desalted by gel filtration on Sephadex G-25 in 0.1 M acetic acid and lyophilized.

Native RNase was the phosphate-free, lyophilized grade from Worthington Biochemical Corp. which had been further purified by chromatography using the same procedure as in Figure 1.

All the samples used for spectral measurements had been subjected to gel filtration on Bio-Gel P60, as described below.

Gel Filtration. A column of Bio-Gel P60 (−400 mesh), 2.5 cm in diameter and 83 cm long, was equilibrated with 0.1 M ammonium bicarbonate at room temperature. Samples of each protein applied to the column contained 0.6 μmol of protein in 3.0 mL of water. The flow rate was about 17 mL/h, and the absorbance at 275 nm was monitored continuously. The proteins were recovered by lyophilization.

Circular Dichroism. All samples were dissolved in 0.05 M phosphate buffer, pH 7 (Fisher, lot 775579), and were filtered through a Millipore No. HATF 01300 filter prior to use. The concentrations of all the proteins were determined spectrophotometrically by means of the molar absorbance of 9200

[1] Abbreviations used: RNase, bovine pancreatic ribonuclease A, irrespective of the state of its cysteine residues; BPTI, bovine pancreatic trypsin inhibitor; HFIP, hexafluoro-2-propanol; CD, circular dichroism.

at 275 nm, determined by Anfinsen et al. (1961) to apply to both native and fully reduced RNase. Values of the circular dichroism were determined with a Cary 60 dichrograph having a Model 6001 circular dichroism attachment. The spectrophotometer was calibrated with camphorsulfonic acid, θ = 7840 deg cm^2 dmol^{-1} at λ 290 nm. The protein concentrations of the samples were adjusted to be within the range 0.5–1.5 absorbance units at 275 nm. Spectra were measured in silica cuvettes with path lengths varying from 0.05 to 20 mm. The spectra were measured at least twice on different days with newly prepared samples and the results averaged. The base line was measured for each spectrum. The circular dichroism is reported as the mean residue ellipticity, calculated from the relationship $\theta = hs/lc$ where h is the magnitude of the circular dichroism band in fraction of the full scale, s is the sensitivity of the instrument in degrees per full scale, l is the path length of the cuvette in centimeters, and c is the mean amino acid residue concentration in moles per liter.

Raman Spectra. Spectra excited by Ar^+ radiation at 488 nm (100 mW at the sample) from a Coherent Radiation 52G laser were recorded in the range 450–1600 cm^{-1} with a Spex Ramalog Model 4 Raman spectrophotometer. The spectral slit width was 4 cm^{-1}, with a time constant of 12 s and a scanning rate of 0.07 cm^{-1}/s. Liquid samples with protein concentrations from 5% to 8% (w/v) dissolved in unbuffered distilled water (except for fully reduced RNase, which was dissolved in 0.1 M Tris buffer, pH 7.5) were contained in silica capillaries of 1 mm inner diameter. All spectra have been replotted, after correction for the different fluorescent background of each sample, to a common horizontal background (Figure 6). The precision of the relative intensities is estimated to be about 10%.

Results

Trapped Folding Intermediates of RNase. The intermediates which accumulate during refolding of reduced RNase were trapped by rapidly alkylating all free thiol groups with iodoacetate, so that disulfide bond formation, breakage, and rearrangement would not occur (Creighton, 1978). This makes possible the resolution of the complex mixtures trapped at various times of refolding by ion-exchange chromatography (see Figure 1).

The RNase molecules elute essentially in the order of the number of disulfide bonds they contain. Trapped fully reduced RNase, R, with no disulfide bonds, elutes first from the column, followed by single major peaks of the one- and two-disulfide intermediates, designated I and II, respectively. The three-disulfide intermediates are eluted in two partially resolved peaks, designated IIIA and IIIB. Each of the peaks I, II, IIIA, and IIIB has been concluded to contain a number of species with different pairings of cysteine residues in disulfide bonds (Creighton, 1979).

The four-disulfide species have been resolved into three peaks. The fully resolved last peak, N, contains only correctly refolded RNase, with four correct disulfide bonds. The preceding partially resolved peaks, IVA and IVB, contain primarily molecules incorrectly folded, with two or more incorrect disulfide bonds, but also contain a significant fraction of molecules with many of the properties of native RNase, but much more susceptible to unfolding by urea. Peaks IVA and IVB were not so well-resolved previously and were treated as a single peak, IV (Creighton, 1979).

Compactness of the Polypeptide Chain Measured by Gel Filtration. Gel filtration through molecular sieves of the appropriate pore size is a convenient measure of the overall relative dimensions of the polypeptide chain (Halvorson & Ackers, 1974; Acharya & Taniuchi, 1976, 1977). Trapped RNase with no disulfide bonds, R, which is well-known to be highly unfolded (Tanford, 1968), was observed to elute much earlier than native RNase (Figure 2), presumably due to the much greater dimensions of the former. The RNase intermediates I, II, IIIA, and IIIB were eluted as symmetrical peaks at the intermediate positions indicated in Figure 2. Increasing numbers of disulfide bonds produce a decrease in the dimensions of the polypeptide chain by covalent cross-linking, but no significant fraction of molecules in these mixtures had an elution position approaching that of native RNase.

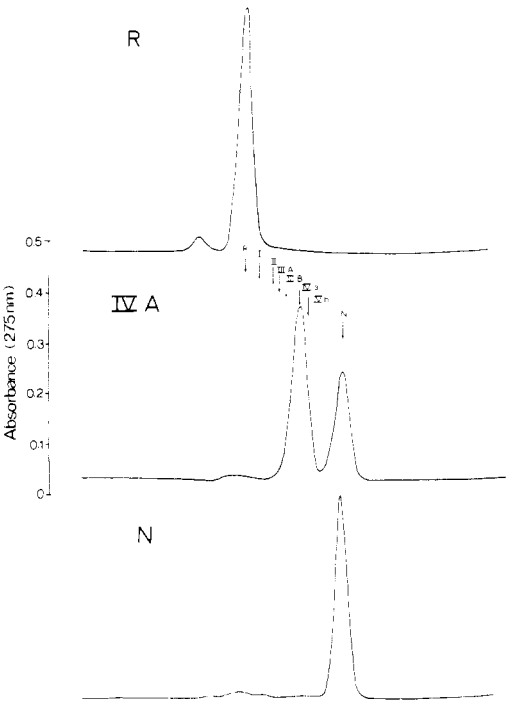

FIGURE 2: Gel filtration of RNase folding intermediates. The column was Bio-Gel P60 in 0.1 M ammonium bicarbonate, and the elution profiles were determined by the absorbance at 275 nm. (Top) Profile of trapped reduced RNase, R. (Middle) Elution profile of species IVA, showing the resolution into noncompact (IVa) and native-like peaks (positions of the maxima of the peaks of the other species indicated by arrows). The positions indicated for IVa and IVb are of the major, noncompact peaks in IVA and IVB, respectively. (Bottom) Correctly refolded RNase, N.

In contrast, mixture IVA gave the bimodal profile illustrated in Figure 2, with a substantial fraction of the molecules having the compactness of native RNase. This fraction is the same as that found previously to be very similar to native RNase, but to be unfolded much more readily by urea (Creighton, 1979). It was subsequently found to have only three disulfide bonds, so is designated IIIn. Its anomalous chromatographic behavior is not unprecedented, as conformation and relative positions of charged groups have been observed to affect chromatographic mobility (Creighton, 1975). Mixture IVB gave a similar profile, but contained a much smaller proportion of this compact protein species. The noncompact protein peaks obtained upon gel filtration of IVA and IVB were designated IVa and IVb, respectively.

Rearrangement of Disulfides and Changes in Compactness. The possibility of the noncompact species rearranging their disulfide bonds, upon addition of a thiol catalyst, so as to attain a stable compact conformation, was then investigated. The various trapped intermediates of RNase isolated above were incubated at 25 °C overnight at a concentration of 4 μM in 0.1 M ammonium bicarbonate containing 1.4 mM mercaptoethanol, as thiol catalyst, and 2.5 mM hydroxyethyl disulfide,

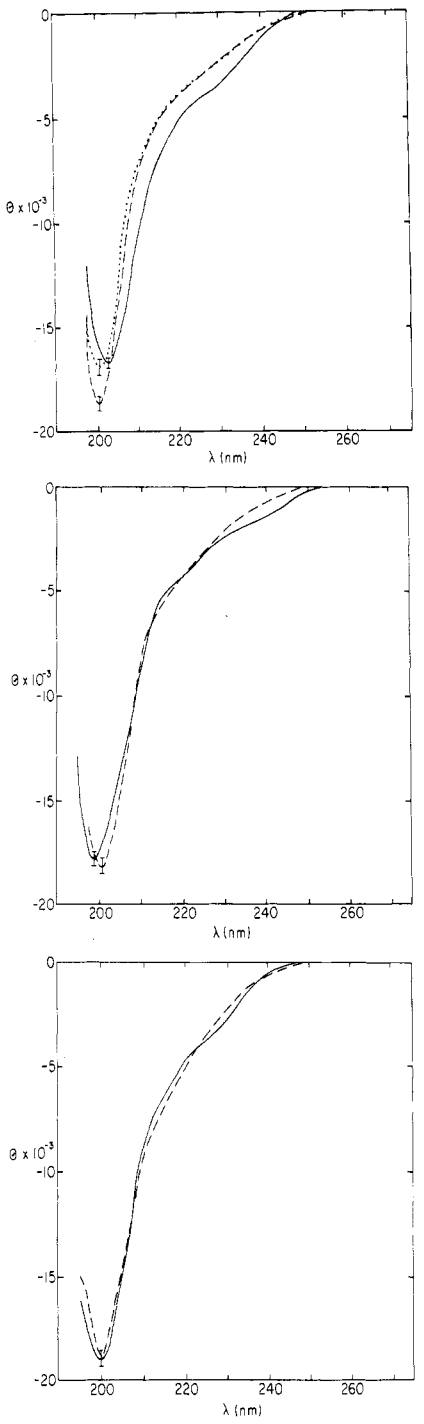

FIGURE 3: CD spectra (mean residue ellipticity) of reduced RNase and refolded intermediates in 0.05 M phosphate buffer, pH 7. (a, top) (—) R; (---) I; (···) II. (b, middle) (—) IIIA; (---) IIIB. (c, bottom) (—) IVa; (---) IVb.

to ensure thermodynamic stability of protein disulfides. The compactness of the RNase molecules was then measured by gel filtration, as above.

Native RNase was unaffected by this procedure, while intermediates IVa and IVb were quantitatively converted to native-like RNase, thereby illustrating the unique stability of the native pairings of disulfide bonds and the efficacy of the disulfide rearrangement procedure. One-disulfide intermediates would not be expected to rearrange disulfide bonds intramolecularly, and the elution profile of I was found to be unchanged. The elution profiles of II, IIIA, and IIIB were also unchanged, indicating that alternative disulfide pairings could not produce a more compact conformation which was

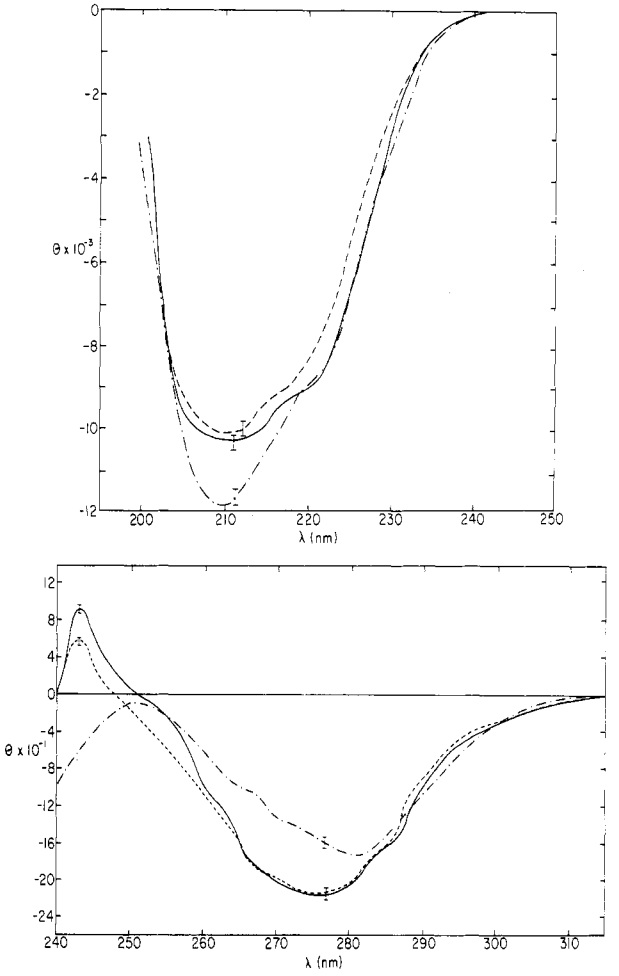

FIGURE 4: CD spectra (mean residue ellipticity) of native RNase and enzymatically active refolded RNase in 0.05 M phosphate buffer, pH 7. (a, upper) In the far-UV; (b, lower) in the near-UV. (—) Native RNase; (---) refolded RNase; (···) IIIn.

also more favorable energetically.

Circular Dichroism Spectra. The circular dichroism spectra of native and fully reduced RNase are well-known to be markedly different, due to their very different conformational properties (Simons & Blout, 1968; Schaffer, 1975; Takahashi et al., 1977). The spectra measured here of these limiting conformational states were very similar to those reported by others and serve as standards with which to compare the spectra of the RNase folding intermediates.

The spectrum of reduced RNase has only a deep minimum at about 200 nm (Figure 3a), similar to that expected of a disordered polypeptide chain. In contrast, native RNase has a spectrum with a smaller, complex minimum at 210 to 220 nm, a small positive peak at 242 nm, and a complex minimum in the near-UV region (Figure 4a,b) as a result of the ordered conformation of the backbone and of the presence of tyrosine and disulfide side chains in asymmetric environments in the folded state (Strickland, 1972; Woody, 1978).

The RNase intermediates I, II, IIIA, IIIB, IVa, and IVb gave spectra very similar to that of R (Figure 3), with no near-UV circular dichroism and only a minimum at 200 ± 1 nm of very similar intensity. The shapes of the curves differed somewhat in the region 210–250 nm, but this was not related to the number of disulfide bonds. For example, R has a broader spectrum than I or II, and that of IVa is broader than that of IIIA or IIIB. Circular dichroism thus reveals no stable partially ordered conformation in these species. Similar results have been reported by Konishi & Scheraga (1980).

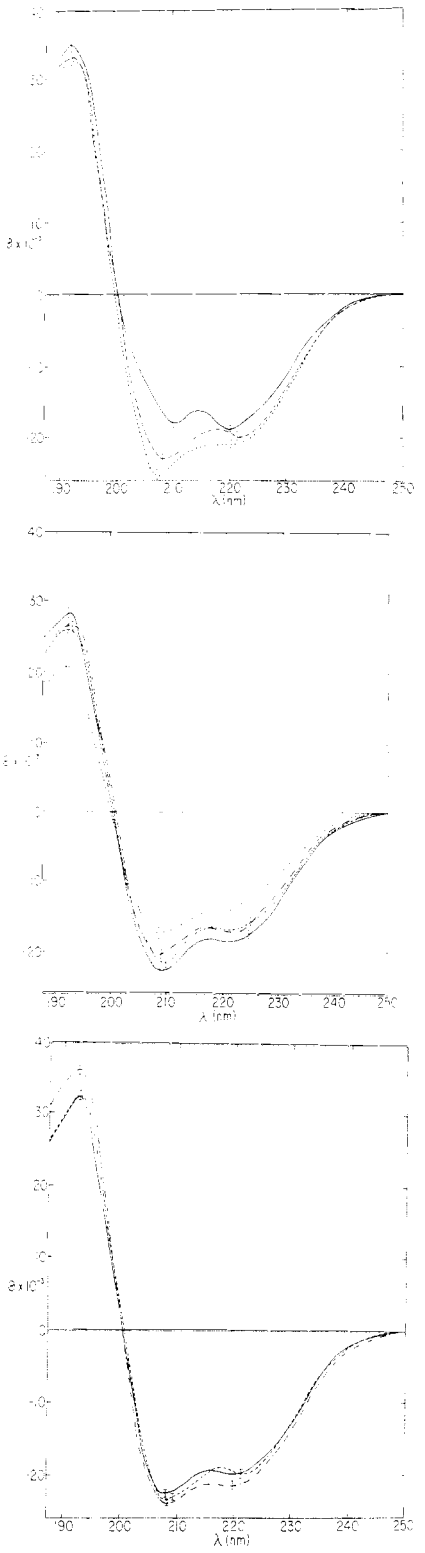

FIGURE 5: CD spectra (mean residue ellipticity) of native, fully refolded, intermediate and fully reduced RNases in 0.05 M phosphate buffer, pH 7, HFIP mixture 1:1. (a, top) (—) R; (---) I; (···) II. (b, middle) (—) IIIA; (---) IIIB; (···) IVa; (-·-) IVb. (c, bottom) (—) native RNase; (---) refolded RNase; (-·-) IIIn.

In contrast, the species IIIn, found to be compact by gel filtration (Figure 2) and native-like by other criteria (Creighton, 1979), gave a spectrum similar to that of native RNase, but with slight deviations throughout, indicating corresponding differences in conformation. The spectra of native and refolded RNase were essentially identical, including the sensitive positive peak at about 242 nm, although the far-UV spectrum of refolded RNase was somewhat narrower

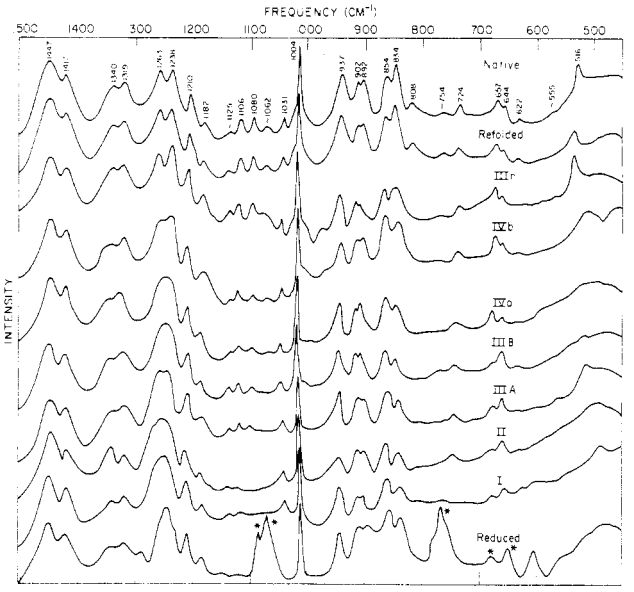

FIGURE 6: Raman spectra of aqueous solutions of native, fully refolded, intermediate, and reduced RNases, replotted to a horizontal background. Spectroscopic parameters as given in text. Asterisks in the bottom spectrum (R) indicate lines due to the Tris buffer.

than that of native protein.

Induction of Helicity by HFIP. As the circular dichroism spectra of most of the RNase folding intermediates gave no indication of partially ordered conformation, their conformational flexibilities were examined by the ability of the solvent hexafluoro-2-propanol (HFIP) to induce helicity (Timasheff, 1970; Parrish & Blout, 1971, 1972). Circular dichroism spectra measured in 33% (v/v) HFIP (pH maintained within the range 6.2–6.6 by phosphate buffer) are illustrated in Figure 5. Similar spectra were obtained with 10%, 50%, and 67% HFIP. Spectra very like those of α-helical polypeptides were obtained with all the RNase derivatives, although the intensities of the CD bands were only about half those observed with synthetic polypeptides (Parrish & Blout, 1971). The intensity was somewhat lower with intermediates IIIA, IIIB, IVa, and IVb, suggesting that the disulfide bonds limit somewhat the ability to assume an α-helical conformation. Similar spectra were also obtained with native and refolded RNase and with intermediate IIIn (Figure 5c), suggesting that the normal folded conformation has been disrupted by HFIP and replaced by a helical conformation.

Raman Spectra. The Raman spectra of aqueous solutions of the various forms of RNase, replotted to a horizontal base line, are shown in Figure 6. The Raman spectra of native RNase and of reversibly thermally unfolded RNase have been discussed previously by Lord & Yu (1970) and by Chen & Lord (1976), respectively. We use the same framework for consideration of the spectra as Chen & Lord (1976).

The Amide III Contour, 1220–1300 cm^{-1}. Because of the relatively strong and broad water band centered at about 1640 cm^{-1}, the information provided by the amide I contour in the range 1640–1680 cm^{-1} is much less clear-cut than that of the amide III region. For all but the refolded and IIIn proteins, the amide III region shows a peptide backbone more disordered than the thermally unfolded molecule at 70–75 °C. There is some variation in amide III half-widths and some trace of doublet structure in the spectrum of IVB, but the other contours peak in the neighborhood of 1250 cm^{-1}, at which point in the spectrum the elimination of helical and pleated-sheet structure can be followed thermally (Chen & Lord, 1976). In any event, by this criterion there is more residual order in

reversibly denatured RNase at 70 °C than in any of the reoxidized fractions except the fraction IIIn, which appears to have as much helical and pleated-sheet composition as native RNase. Completely reduced RNase shows an unexpectedly narrow amide III band (half-width roughly 34 cm^{-1} compared with more nearly 44 cm^{-1} in the various oxidized forms), and a marginally significant shift to lower frequency (1248 cm^{-1}). This suggests the presence of a certain amount of β structure in the fully reduced enzyme.

Behavior of the Tyrosine Doublet at 855 and 830 cm^{-1}. The intensity ratio, I_{854}/I_{830}, of this doublet has been shown to indicate the effect of hydrogen bonding on the tyrosine hydroxyl group (Siamwiza et al., 1975). When the hydrogen of the hydroxyl group is strongly bonded to a negatively charged acceptor such as CO_2^-, the above ratio is well below unity, whereas with weak bonding it may be 1.5 or higher. The completely reduced sample and all of the reoxidized forms except the correctly refolded IIIn show a doublet intensity ratio of 1.2 or higher (because of the base-line uncertainty, precise values are not easy to determine). This indicates that the six tyrosines in the reoxidized forms are mostly or entirely weakly hydrogen bonded to acceptors such as the oxygen of the aqueous solvent. Only in the partially refolded form IIIn does one find a smaller intensity ratio (1.0). In the native spectrum the ratio is about 0.85 at room temperature, and thermal denaturation raises this to unity at 60 °C and to 1.5 at 70 °C (Chen & Lord, 1976). Thus the tyrosine doublet affirms the disordered nature of the peptide backbone in all but the IIIn form; in the latter it appears that one of the three strongly H-bonded tyrosines of native RNase has lost its strong hydrogen bond.

Disulfide Frequency. As noted earlier (Lord & Yu, 1970; Chen & Lord, 1976), the disulfide frequency in native RNase at room temperaure and pH 5–7 is higher than in other proteins (516 vs. 509 cm^{-1}). The relative sharpness of this line (half-width 14 cm^{-1}) suggests that the geometries of the four S–S bonds in native RNase are closely similar. It might be supposed, and indeed was expected, that in the spectra of the partially oxidized proteins the integrated intensity of the line, if not its peak height, might be a measure of the number of reoxidized disulfide bonds. Unfortunately, the variability of the frequency in the different S–S groups makes it difficult to measure the area of the S–S line in the presence of the background due to H_2O, or even to say qualitatively in several cases that the S–S frequency is present. Only in the spectrum of the IIIn sample is the S–S frequency found at the native location of 516 cm^{-1}.

Discussion

The conformational properties of the RNase molecules with different numbers of disulfide bonds trapped during the refolding of the reduced protein are of interest in themselves and are particularly relevant to the question of the role of protein disulfide bonds in stabilizing folded conformations. They are also pertinent to elucidation of the conformational forces that direct disulfide bond formation during refolding. Disulfide formation is the only means, at present, of trapping the transient, unstable intermediates in protein folding transitions, and under proper conditions the varying tendencies of the cysteine residues to form disulfide bonds should reflect the conformational properties of the polypeptide chain at the various stages of folding. Any partially ordered conformational properties should also be evident in the trapped intermediates, as whatever conformation favors formation of a particular disulfide bond should, conversely, be favored to a similar extent in the intermediate with that disulfide bond.

The RNase intermediates studied here were isolated after trapping them during refolding of the fully reduced protein in an attempt to elucidate the folding pathway and to compare it with that determined for bovine pancreatic trypsin inhibitor, BPTI (Creighton, 1978). The RNase pathway was found to be complex and ill-defined, as has also been observed by others (Anfinsen et al., 1961; Hantgan et al., 1974; Takahashi et al., 1977), in that a large number of disulfide intermediates accumulate. The pathway converges from the initial randomness of the disordered reduced protein to unique disulfide bonds of the native protein much less rapidly for RNase than for BPTI. The mixture of single-disulfide intermediates, for example, shows no apparent predominance of any of the 28 possible disulfide pairs, while in BPTI only two such intermediates predominate. The two- and three-disulfide intermediates may be produced more restrictively than would be expected on a straight probability basis, but with a distribution that leads preferentially to the formation of incorrect four-disulfide species. Correctly refolded RNase appears to be formed only after relatively slow (or less probable) disulfide rearrangements in the three-disulfide intermediates. At least some of the molecules fold through the native-like three-disulfide intermediate IIIn, but whether this is an obligatory intermediate is not yet known.

Although there are certain similarities between the folding pathways of reduced RNase and reduced BPTI (Creighton, 1977–1979), a striking difference is the far fewer constraints on disulfide bond formation in the case of RNase. This is undoubtedly due to the much larger number of conformational degrees of freedom in RNase at the initial stages of folding, as indicated by the apparent absence of partially ordered conformations in the initial intermediates. The gel filtration, circular dichroism, and Raman studies have failed to detect partially ordered conformations in all the RNase intermediates with 0 to 4 disulfide bonds (other than intermediate IIIn and the correctly refolded N), and their flexibility is shown by the ease with which the solvent HFIP induces a helical conformation. These studies support and extend the similar previous findings by polyacrylamide gel electrophoresis, enzyme activity, UV absorbance, urea–gradient electrophoresis, and binding to antibodies against native or reduced RNase (Creighton 1977, 1979). In contrast, the trapped BPTI intermediates clearly have partially ordered conformation by the same criteria (Kosen, 1978; Kosen et al., 1980).

A stable partially ordered conformation appears during refolding of reduced RNase only when a minimum of three correct disulfide bonds are formed, as in intermediate IIIn. This species is enzymatically active and native-like in most aspects of its conformation, although the circular dichroism and Raman spectra indicate a number of small deviations from the normal native conformation. This appears to be the only three-disulfide species of RNase which can have a stable compact conformation, as no others have been detected to accumulate during unfolding or refolding, and no others were formed when disulfide rearrangement was permitted in the trapped three-disulfide intermediates. This is in contrast to lysozyme, where all four three-disulfide species lacking individually each of the four native disulfides have been reported to have a stable compact conformation (Acharya & Taniuchi, 1976, 1977).

Intermediate IIIn has been observed to accumulate only during refolding, not during unfolding of native RNase, under the conditions used here. If it is an intermediate in unfolding, it must be very susceptible to further disulfide reduction under the conditions used here, so that it does not accumulate.

Recent work (Creighton, 1980) indicates that the broken disulfide bond in IIIn is that between cysteines 40 and 95, which is consistent with the foregoing.

The spectral properties of the other trapped intermediates from R to IVb are very similar. While in certain proteins randomly oriented chromophores of aromatic amino acids may exhibit Cotton effects which do not cancel (Strickland, 1974), such a possibility in the case of these intermediates is entirely excluded, since no CD band is observed in the spectral region 250–320 nm. If one assumes that randomly oriented aromatic amino acids make a negligible contribution to the intensity of the far-UV Cotton effects of intermediates, one may conclude that slight differences between their CD curves as well as the varied broadness of the amide III bands in their Raman spectra imply minor differences in the secondary structure of these proteins. Though the conformations of the trapped intermediates are essentially random, presumably the distribution of the ϕ, ψ angles characteristic of different secondary structures varies through the series from R to IVb. Thus certain distributions of peptide geometry may exhibit moderately intense $n \rightarrow \pi^*$ transitions, which in fact are mainly responsible for the observed differences in the CD curves in the range 220–260 nm.

This situation is completely changed in the case of refolded, native, and IIIn RNase. Due to rather modest content of α-helical conformation in native RNase and predominance of antiparallel β structure and β turns, the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of the α helix are strongly overlapped and even partially obscured by the electronic transitions of the other two conformations. The somewhat larger Cotton effect of IIIn at about 208 nm in comparison with native or refolded RNase is presumably due to the lack of "aromatic contribution" to the far-UV spectral region and does not reflect any difference in conformation of IIIn in comparison with refolded or native RNase. The profile of the CD band at about 242 nm of native RNase clearly shows that the Cotton effects of the tyrosines are much larger in this spectral region and their real maximum is somewhere below 240 nm. However, the $n \rightarrow \pi^*$ transitions of the α helix are of the opposite sign, and about 25% of this conformation is sufficient to overshadow almost completely the electronic transitions of the six tyrosines.

While native and refolded RNase exhibit closely similar CD and Raman spectra, RNase IIIn differs significantly from these two in the near-UV region as well as in the intensity ratio of the tyrosine doublet in the Raman spectra. The intensity of the set of CD bands in the range 250–320 nm is smaller than in native and refolded RNase, which must be related to considerable variability in the orientation of the long axis of the tyrosine aromatic rings in the tertiary structure of RNase IIIn. This variability leads to cancellation of the Cotton effects of these variously oriented aromatic rings; without such variability the CD spectrum in the near-UV region of species IIIn should not differ much from the CD spectrum of native RNase. The same conclusion is suggested by the ratio of unity for I_{850}/I_{830} in the Raman spectrum of RNase IIIn, whereas in native and refolded RNase this ratio is 0.8.

Finally, the circular dichroism and Raman spectra of refolded RNase leave little doubt that it has a conformation very close to that of native RNase. This does not necessarily reflect a refolding procedure more efficient than those used by others who found differences in refolded RNase (Beychok, 1966; Pflumm & Beychok, 1969; Isemura et al., 1968), but rather is probably a result of purifying the correctly refolded RNase by ion-exchange chromatography (Figure 1), removing molecules with incorrect disulfide bonds and those which may have been modified covalently (Creighton, 1979).

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