A Study on the Conformation and Conformational Stability of Ribonuclease A and Its Peptic Derivative, Des-(121–124)-ribonuclease†

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ABSTRACT: We have determined the near-ultraviolet circular dichroic spectrum (235–315 nm) and the ultraviolet difference absorption spectrum of ribonuclease A (RNase A) and its peptic derivative, des-(121–124)-RNase A (RNase P), which is missing the four C-terminal residues and retains no more than a few per cent of the enzymic activity of RNase A. The circular dichroic spectra of both proteins were resolved into gaussian parameters and the rotational strengths of the resolved bands (due mainly to tyrosines and disulfides) were found to be very similar in the two proteins. Also, the denaturation difference spectrum was almost identical, thus indicating that in each native protein the same number of tyrosyl groups are inaccessible to solvent. The circular dichroic spectrum of RNase P between 195 and 235 nm was also obtained and compared to the known spectrum of RNase A. The general shape of the spectra of the two proteins was similar, although the ellipticity of RNase P at the extrema was somewhat lower than that of RNase A. These results suggest close similarity in the conformation of the two proteins. Measurements of the reversible changes in the near-ultraviolet circular dichroic spectrum due to different concentrations of the denaturant, guanidine hydrochloride, enabled us to estimate the conformational free energy of unfolding, ΔG_0, of each protein in the absence of denaturant using the two-state transition theory, which appears suitable for both RNase A and the peptic derivative. In a buffer containing 10 mM phosphate, ΔG_0 was estimated to be 14.4 ± 0.3 kcal/mole for RNase A and 10.1 ± 0.7 kcal/mole for RNase P. Thus, the removal of only four residues from the C terminus of RNase A reduces ΔG_0 by about 30%, or 4.3 ± 1 kcal/mole. These results clearly demonstrate the importance of the C-terminal region in protein folding, and suggest that a stable tertiary structure develops only during the terminal stages of biosynthesis.

Bovine pancreatic ribonuclease is a well-characterized enzyme, and its structure (Smyth *et al.*, 1963; Kartha *et al.*, 1967; Wyckoff *et al.*, 1967, 1970) and properties, as well as those of various enzymic and chemical derivatives, have recently been reviewed (Richards and Wyckoff, 1971). One interesting enzymic derivative, described by Anfinsen (1955, 1956), was prepared by the limited digestion of RNase A[1] with pepsin. The product was missing the four C-terminal residues and appeared to be completely inactive. Using a highly purified derivative, Lin (1970) later showed that the activity of RNase P, albeit very low, was not zero; however, removal of Phe-120 by carboxypeptidase did produce an inactive derivative. Since the spectral properties (Sela and Anfinsen, 1957; Taniuchi, 1970) and the hydrodynamic properties (Anfinsen, 1956) are altered only slightly by the removal of the C-terminal tetrapeptide, it appears that the overall conformation of RNase P is quite similar to that of RNase A. From these results one can conclude that the large reduction in enzymic activity is due to a slight rearrangement at the active site, and not to a major conformation change.

Lin (1970) found that the transition temperature of RNase P was 16.5° lower than that of RNase A, thus showing a decreased stability of the peptic derivative. Taniuchi (1970) observed that reoxidation of reduced, unfolded RNase P yielded a product with a circular dichroic (CD) spectrum typical of a disordered protein. Also, chemical analysis of the reoxidized protein indicated the occurrence of several mismatched, intramolecular disulfide bonds. It is known that reoxidation of reduced RNase A leads to a return of enzymic activity (Anfinsen and Haber, 1961), and it was thus concluded that the globular structure of RNase A could not be formed until the polypeptide chain had been synthesized beyond residue 120.

Since the conformation of RNase P with intact disulfide bonds reflects a slightly perturbed RNase A structure, this seems to be an ideal derivative for investigating the effects of small changes in primary structure on the conformation and conformational stability of proteins. Numerous reports have appeared on the thermal unfolding (Hermans and Scheraga, 1961a; Holcomb and Van Holde, 1962; Brandts and Hunt, 1967; Klee, 1967), acid unfolding (Hermans and Scheraga, 1961b; Bigelow and Krenitsky, 1964), urea unfolding (Foss and Schellman, 1959; Nelson and Hummel, 1962; Barnard, 1964; Bradbury and King, 1969), and guanidine hydrochloride unfolding (Salahuddin and Tanford, 1970) of RNase A. Consequently, similar studies on RNase P

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[1] Abbreviations used are: RNase A, the major bovine pancreatic ribonuclease; RNase P, to be consistent with the nomenclature of the other ribonuclease derivatives, we propose this form for the (limited) peptic derivative of RNase A, *i.e.*, des-(121–124)-RNase A; RNase S, the derivative of RNase A produced by (limited) digestion with subtilisin (which cleaves between residues 20 and 21 or between 21 and 22); Gdn·HCl, guanidine hydrochloride.

should be informative with regard to the conformation and stability of the derivative, and should elucidate the nature and importance of the interactions at the C-terminal region of RNase A.

We have investigated the *difference* in the conformational stability of RNase P and RNase A by monitoring the *reversible* changes in the CD spectrum and the ultraviolet difference absorption spectrum of each protein at various guanidine hydrochloride concentrations. From these data the conformational free energy of each protein can be estimated (Tanford, 1970). From a model of ribonuclease, assembled using the coordinates of Wyckoff *et al.* (1970), the interactions of the four C-terminal amino acid residues with the remainder of the protein have been determined, and from these data we have attempted to account for the observed difference in the conformational free energy.

Experimental Section

Materials. Bovine pancreatic RNase A was obtained from Sigma Chemical Co. (type XII-A, lot no. 39B-8090) and had a stated activity of 100 Kunitz units/mg. Pepsin, with a stated activity of 2530 units/mg (lot no. PM9FD), DFP-treated carboxypeptidase A with a measured activity of 45 units/mg (assayed using hippuryl-L-phenylalanine as described by Folk and Schirmer, 1963), and yeast RNA (type XI, lot no. 98B-8050) were Worthington products. Spectrophotometric grade guanidine hydrochloride was from Heico, Inc. (Delaware Water Gap, Pa.), and the other salts were certified ACS Fisher products. SE-Sephadex C-25 was obtained from Pharmacia and Bio-Gel P-4 (50–100 mesh) from Bio-Rad Laboratories.

Preparation of RNase P. The procedure of Anfinsen (1956), as modified by Lin *et al.* (1968), was used to remove the RNase A C-terminal tetrapeptide and to separate RNase P from this and the unreacted RNase A. Typically 200 mg of RNase A was dissolved in 20 ml of water, and 1 N HCl was added to give a pH of 1.8. Pepsin, 0.8 mg/ml in water, was added to give a RNase A:pepsin ratio of 5000:1 by weight. The mixture was incubated for 10 min at 37° with occasional stirring. The digestion was terminated by the addition of 2 ml of 1 M sodium phosphate (pH 6.45). The mixture was applied directly to a 2.5 × 40 cm column of SE-Sephadex equilibrated with 0.13 M sodium phosphate (pH 6.45) at 4°. The column was developed with the same buffer at a flow rate of 93 ml/hr and individual fractions (4.7 ml) were monitored at 230 or at 280 nm using a Beckman DU-2 spectrophotometer. RNase P, which elutes as the last major peak (Lin, 1970), was pooled, lyophilized, and desalted on a 2 × 58 cm column of Bio-Gel P-4, equilibrated with 5% acetic acid. The protein peak was pooled, lyophilized, and stored in powder form at −15°. The average yield was 25%. For N-terminal sequence analysis, 10 mg of RNase P was oxidized with performic acid at 0° using the method of Hirs (1956).

Amino Acid Analysis and Sequence Determination. About 0.34 mg of protein was hydrolyzed in 6 N HCl for 20 hr at 110° with norleucine as an internal standard. The analysis was performed on a Beckman 120 amino acid analyzer (Spackman *et al.*, 1958). About 5 mg of performic acid oxidized RNase P was analyzed for N-terminal residues by automatic Edman degradation (Edman and Begg, 1967) using the Beckman automatic sequenator with the Quadrol double-cleavage program, followed by the gas–liquid chromatographic method of Pisano and Bronzert (1969) for identification of the phenylthiohydantoin derivatives. All phenylthiohydantoin derivatives were reexamined after silylation with *N,O*-bis(trifluoroacetyl)acetamide.

Assays. Enzymic assays were performed as described by Anfinsen *et al.* (1954) and Kalnitsky *et al.* (1959) at pH 5 using yeast RNA as substrate. At zero time 1 ml of 1% RNA in 0.1 M acetate buffer (pH 5.0) was added to a 1-ml solution containing 2–9 μg of enzyme in 0.1 M acetate buffer (pH 5.0) at 37°, and incubated for 4 min with occasional agitation. Then 1 ml of uranyl acetate (0.75%) in 25% perchloric acid was added and the mixture was placed in an ice bath. Following this the mixture was centrifuged for 5 min at 700*g*. A 0.1-ml aliquot from the supernatant was immediately diluted to 3 ml with water and the absorbance read at 260 nm in a 1-cm cuvet against a blank processed in the same manner but lacking enzyme. Normally three enzyme concentrations were employed.

Solution Preparations. Stock solutions were prepared by dissolving a weighed amount of protein in a known volume of deionized, distilled water to give a solution 5–10 mg/ml. Appropriate dilutions were made into volumetric flasks containing buffers and salts to give the desired protein concentration, generally about 1 mg/ml. All solutions contained 10 mM sodium phosphate which was used both to buffer and to eliminate any artifacts arising from varying amounts of residual phosphate. The protein concentration was determined from the absorption peak at 278 nm using an extinction coefficient of 0.71 and 0.73 absorbance unit per mg per ml per cm for RNase A and RNase P, respectively. The latter value accounts for the reduction in molecular weight due to removal of the C-terminal tetrapeptide; Shugar (1952) has shown that the absorbance change at neutral pH following pepsin digestion of RNase A is less than 2%. Also, we have found from quantitative amino acid analysis on solutions of known absorbance that the extinction coefficient of RNase P is a few per cent greater than that of RNase A. The pH of the solutions was measured with a Radiometer 26 meter.

Spectral Measurements. Circular dichroic spectra were obtained using a Cary 60 spectropolarimeter with the Model 6002 CD attachment. Over the wavelength range of 235–320 nm, the spectra were obtained at protein concentrations of 1–2 mg/ml using 5- and 10-mm path lengths. Below 235 nm cells with path lengths of 0.5, 1, and 5 mm were used, and the protein concentration was in the range of 0.1–1 mg/ml. In all cases the full scale was 100 mdeg, the automatic slit control was used, and the time constant was either 3 or 10 sec. Baselines were obtained before and after a series of solution scans and never deviated more than 1–2 mdeg. Unless indicated otherwise, the temperature was 26°.

The molar ellipticity, $[\theta]$, in the conventional units of (deg cm^2)/dmole, was calculated using the eq 1, where θ is the

$$[\theta] = \theta/(10dm) \quad (1)$$

ellipticity in millidegrees, d the path length in centimeters, and m the residue molarity, obtained using a value of 110.5 and 111.1 for the average residue molecular weight of RNase A and RNase P, respectively. The resolution of the spectrum into the minimum number of gaussian curves required for a good fit was achieved using the Hewlett-Packard 9100B programmable calculator with the extended memory and plotter. The rotational strength, R (cgs units), of each resolved CD band was determined from the relation (Beychok, 1967)

$$R = 1.234[\theta^\circ](\Delta/\lambda_0) \times 10^{-42} \quad (2)$$

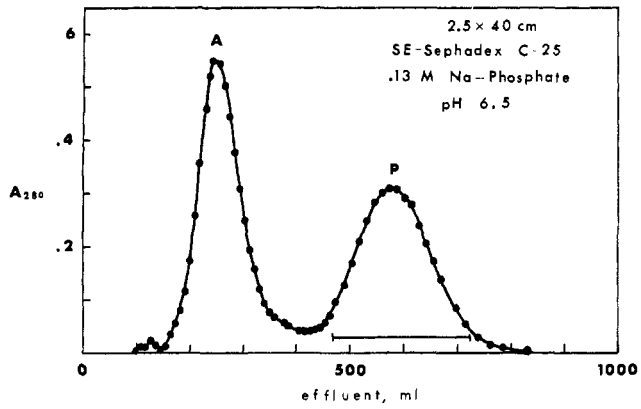

FIGURE 1: Separation of RNase P from unreacted RNase A on a 2.5 × 40 cm sulfoethyl-Sephadex column equilibrated with 0.13 M sodium phosphate (pH 6.5) at 5°. The peptic digestion mixture, after addition of sodium phosphate, was added directly to the column. The C-terminal tetrapeptide elutes ahead of RNase A (Lin, 1970); RNase P was pooled as indicated by the line with brackets.

where $[\theta^\circ]$ is the maximum ellipticity of the resolved band occurring at wavelength λ_0, and Δ is the half-band width (from λ_0 to the wavelength where $[\theta] = [\theta^\circ]/e$).

Equilibrium denaturation ultraviolet difference spectra were measured on an Hitachi Perkin-Elmer double-beam, recording spectrophotometer using 1-cm cuvets and either the 90–110% T or the 50–150% T mode. The reference solution contained either RNase A or RNase P (*ca.* 0.5–1 mg/ml) in 0.1 M KCl–10 mM sodium phosphate and the solution contained the same, as well as guanidine hydrochloride. The kinetics of denaturation and renaturation were followed at the 287-nm extremum using the above conditions.

Results

Characterization of RNase P. A typical elution profile for the separation of RNase P from the C-terminal tetrapeptide and from unreacted RNase A is given in Figure 1. Lin (1970) has shown that under these conditions the tetrapeptide elutes ahead of RNase A. The results of amino acid analysis on RNases A and P were in agreement with the expected values (Hirs *et al.*, 1954; Lin *et al.*, 1968), and showed that the latter contained one mole less of valine, serine, alanine, and aspartic acid from RNase A.

To confirm the specificity of pepsin for the (Phe-120)–(Asp-121) peptide linkage, end-group analysis was performed on our preparation of RNase P. The automated Edman degradation was used for three cycles to obtain the N-terminal sequence of performic acid oxidized RNase P. The sequence was found[2] to be NH_2-Lys-Glu-Thr, in agreement with the known sequence of RNase A (Smyth *et al.*, 1963), and there was no evidence of any N-terminal amino acids other than lysine.

Carboxypeptidase A was used for C-terminal analysis, the digestion being performed at 45° for 20 min. With norleucine as an internal standard, quantitative analysis of the free amino acids, separated from the digest using gel filtration, showed that 1.0 mole each of phenylalanine and histidine was released per mole of RNase P. Occasionally a small amount of alanine, serine, threonine, and valine were also released. These latter residues, invariably less than 10% of the released Phe and His, may be due to a small carry-over of RNase A into the RNase P pool (*cf.* Figure 1), or to some residual proteolytic activity associated with the carboxypeptidase. These data suggest the presence of a single C-terminal residue in the modified protein, since Lin (1970) has shown that carboxypeptidase A removes only Phe-120 and His-119 from RNase P.

The end-group analysis demonstrates that our preparation of RNase P contains a single N-terminal residue (Lys-1),

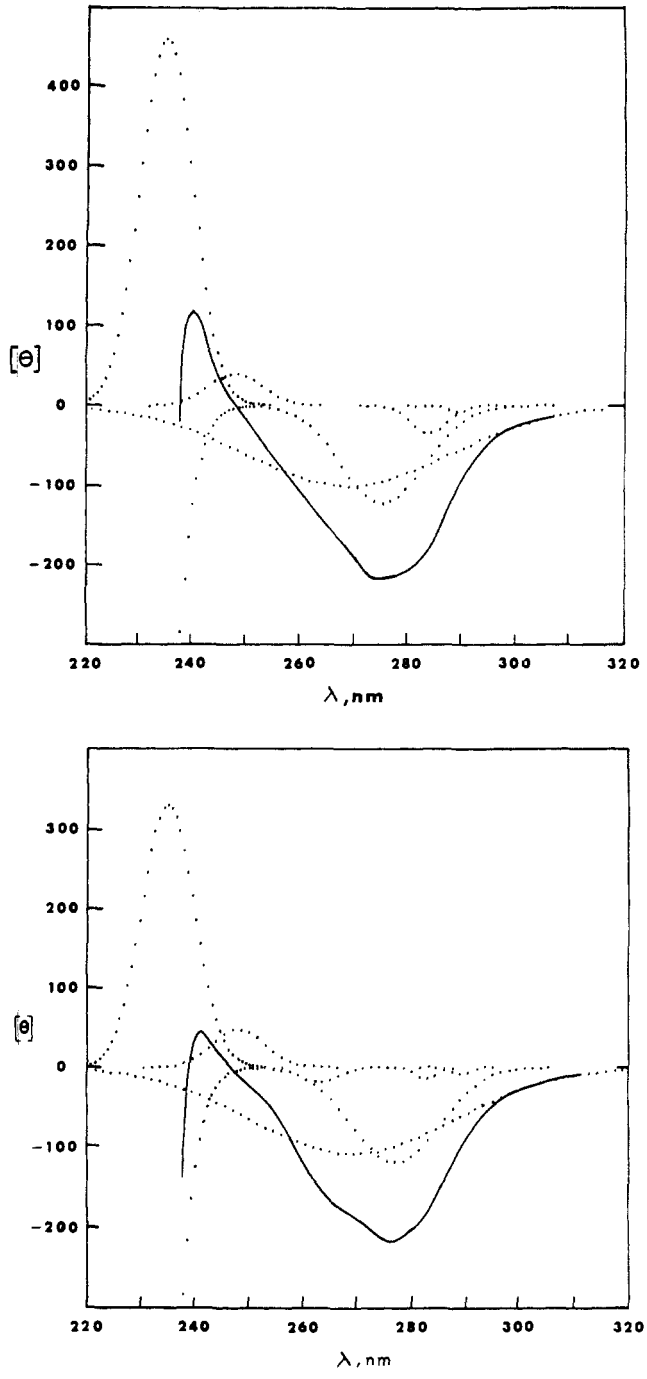

FIGURE 2: The near-ultraviolet circular dichroic spectrum of RNase P (upper) and RNase A (lower) is shown by the solid line which represents the average of several spectra at 26°. The resolved gaussian curves are shown by dotted lines and the guassian parameters are given in Table I. Protein concentrations were in the range 1–2 mg/ml and all solutions contained 10 mM sodium phosphate and 0.1 M KCl.

[2] It is a pleasure to thank Dr. Tadashi Inagami for this determination.

TABLE I: Resolved Gaussian Parameters of the Near-Ultraviolet Circular Dichroic Spectrum of RNase P and RNase A.[a]

Tentative Assignment	RNase P			RNase A		
	λ_0 (nm)	$[\theta°]$	$R \times 10^{42}$	λ_0 (nm)	$[\theta°]$	$R \times 10^{42}$
Tyr	289.2	−8	−(5	289	−8	−0.09
Tyr	284	−35	−	282.7	−14	−0.18
Tyr	275.5	−122	-	277	−119	−6.1
Cys	269	−100	− ,	269	−108	−13.1
Phe				263	−18	−0.42
Phe	248	+40	.47	248	+48	+1.62
Tyr	235	+460	.9	235	+330	+12.0

[a] The rotational stre in cgs units was obtained from the band parameters ; eq 2. The *tentative* assignments to tyrosyl (Tyr) gro phenylalanyl (Phe) groups, or to disulfide bridges (Cy ere made as described in the text, and are based on th sults of Horwitz *et al.* (1969, 1970) and Horwitz and S land (1971). With the exception of the 289-nm band w is believed to result from an inaccessible tyrosine(s), th her tyrosyl bands presumably arise from exposed side ns.

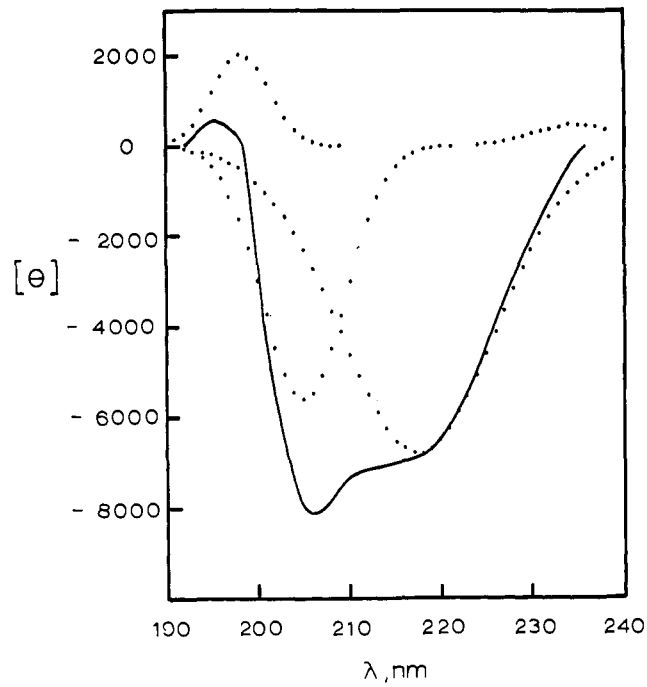

FIGURE 3: The far-ultraviolet circular dichroic spectrum of RNase P at 26° (0.1–1 mg/ml, 10 mM sodium phosphate–0.1 M KCl). The solid line represents the average of several spectra and the dotted lines refer to the resolved gaussian bands (*cf.* Table II).

thus confirming the specificity of pepsin for the (Phe-120)–(Asp-121) peptide linkage and the lack of additional cleavage sites. Our preparation of the modified protein is judged to be at least 95% RNase P, with no internal cleavages, and to contain less than 5% of intact RNase A.

The enzymic activity of RNase P toward yeast RNA was found to be only a few per cent of that of RNase A. RNase P was originally believed to be completely inactive (Anfinsen 1956; Lin *et al.*, 1968; Taniuchi, 1970), although it was recently shown (Lin, 1970) that the derivative will bind inhibitors, and moreover retains about 0.5% of the activity of RNase toward cyclic 2′,3′-cytidylic acid.

Circular Dichroic Spectra. The near-ultraviolet CD spectrum of RNase P and of RNase A is given in Figure 2 along with the resolved components. Curve resolution was aided using the reported λ_0's for the resolved absorbance and CD spectra above 260 nm of RNase A and model compounds at low temperatures (Horwitz *et al.*, 1970). In agreement with the findings of Taniuchi (1970), there is little difference between the CD spectrum of the two proteins over this wavelength region. Since RNase lacks tryptophanyl groups, the near-ultraviolet CD spectrum is due primarily to tyrosyl groups and disulfides. Our spectrum for RNase A agrees with the data of others (Simmons and Glazer, 1967; Simons and Blout, 1968; Simons *et al.*, 1969; Pflumm and Beychok, 1969).

Based on the data of Horwitz *et al.* (1970) and Horwitz and Strickland (1971), tentative assignments of the various bands above 260 nm can be made to the 0–0 transition of either exposed or inaccessible tyrosines, or to disulfides, and these, along with the rotational strengths of the resolved bands, are given in Table I. In order to achieve a good fit to the positive ellipticity around 240 nm, we found it necessary to use two positive bands, at 235 and 248 nm, which overlapped with the negative bands at 269 nm (assigned to disulfides) and at 218–219 nm (assigned to the peptide chromophore). The positive ellipticity near 240 nm appears to result from exposed tyrosyl groups (Simons and Blout, 1968; Pflumm and Beychok, 1969) although there may be contributions from inaccessible tyrosyl groups (*cf.* Timasheff, 1970). Significantly, rat pancreatic ribonuclease which is missing the tyrosyl groups at positions 73 and 76 in bovine RNase A does not have a positive ellipticity at 240 nm (Klee and Streaty, 1970). Consequently, we assign the 235-nm major positive band to one or more of the accessible tyrosyl groups, *i.e.*, Tyr-76, Tyr-92, or perhaps Tyr-115 (*cf.* Lee and Richards, 1971; Richards and Wyckoff, 1971). The 248-nm minor positive band in both proteins, as well as the 263-nm minor negative band in RNase A, is tentatively assigned to phenylalanine residues (Horwitz *et al.*, 1969); however, disulfide groups (Beychok and Breslow, 1968; Urry *et al.*, 1968) and tyrosine residues can also contribute near 250 nm.

In a complicated spectrum such as this, the resolved bands represent a minimum number required for a good fit and certainly do not constitute a unique set. The main impetus for resolving such complicated spectra is that it enables one to quantitatively compare similar spectra, and also it permits one to calculate the entire observed spectrum to within 1–2%. At this level of resolution there is close similarity between the bands of RNase A and RNase P.

In Figure 3 the resolved far-ultraviolet CD spectrum of RNase P is given. Rotational strengths and tentative band assignments are given in Table II. Clearly, these bands represent a definite minimum (Schellman and Lowe, 1968) since complete resolution would necessitate introduction of $n-\pi^*$ and $\pi-\pi^*$ bands associated with each conformation, *e.g.*, α helix, β structure, and non-helix, as well as contributions from aromatic groups and disulfides in this region. For these reasons the far-ultraviolet band parameters in Table II should be viewed simply as curve characterization parameters with little emphasis being placed on a molecular interpretation. The spectrum is in reasonably good agreement with that obtained by Taniuchi (1970), although his

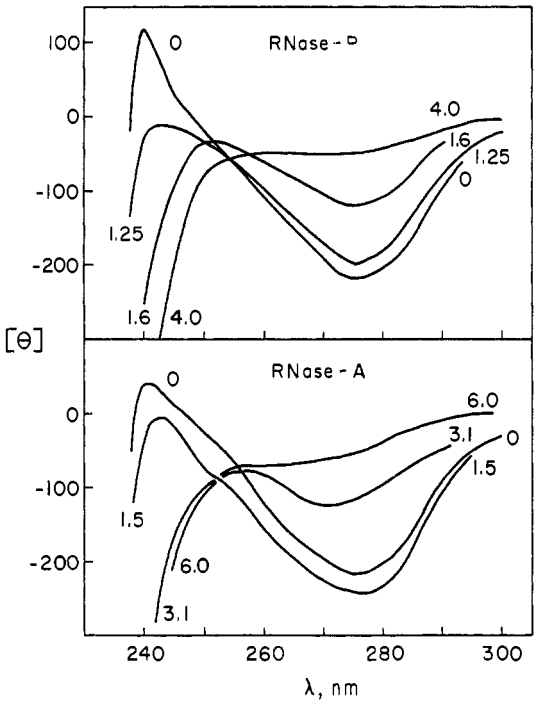

FIGURE 4: The near-ultraviolet CD spectra of RNase P and RNase A at the indicated Gdn·HCl concentration (molarity). The conditions are the same as those in Figure 2.

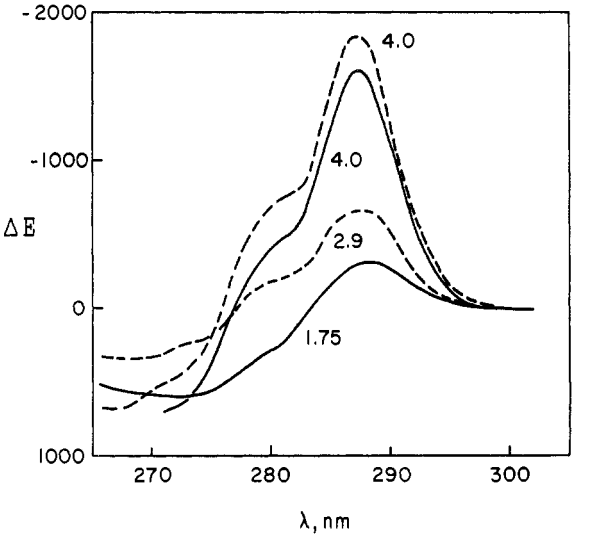

FIGURE 5: The (denaturation) difference spectrum of RNase P (—) and RNase A (----) at the indicated molarity of Gdn·HCl. All solutions contained 10 mM sodium phosphate and the reference solution contained 0.1 M KCl as well. E denotes the molar extinction coefficient.

TABLE II: Resolved Gaussian Parameters of the Far-Ultraviolet Circular Dichroic Spectrum of RNase P.[a]

Assignment	λ_0 (nm)	$[\theta^\circ]$	$R \times 10^{40}$
n–π^*	217.5	−6800	−4.63
π–π^*	205	−5600	−2.12
π–π^*	198.2	+2000	+0.55

[a] The rotational strength in cgs units was determined using the guassian parameters and eq 2. The tentative assignments refer to transitions of the peptide chromophore (*cf.* Beychok, 1967; Schellman and Lowe, 1968). The above bands, along with the data in Table I, give a good fit to the experimental CD spectrum over the wavelength interval 192–320 nm, although as discussed in the text little physical meaning can be attached to the far-ultraviolet bands.

data did not extend below 210 nm. At all wavelengths the ellipticity of RNase P is somewhat less than the corresponding value for RNase A[3] (Schellman and Lowe, 1968; Tamburro *et al.*, 1968a; Pflumm and Beychok, 1969; Klee and Streaty, 1970).

Effect of Guanidine Hydrochloride. The near-ultraviolet CD spectra of RNase P and of RNase A at various concentrations of Gdn·HCl are shown in Figure 4. RNase A is known to unfold in concentrated Gdn·HCl (Bigelow, 1964; Tanford *et al.*, 1967; Nozaki and Tanford, 1967; Salahuddin and Tanford, 1970), and presumably a similar conformation change occurs with RNase P. These data show that unfolding produces significant changes in the near-ultraviolet CD spectrum. Several ultraviolet difference spectra are presented for the two proteins in Figure 5. The difference spectrum is primarily a measure of the inaccessible tyrosyl groups and is seen to be similar for both proteins in 4 M Gdn·HCl.

The ellipticity at 275 nm and at 241 nm is plotted as a function of Gdn·HCl concentration in Figure 6 for RNase A and its peptic derivative. Based on the assignments given in Table I, the ellipticity at 275 nm has about equal contributions from disulfide bridges and exposed tyrosines, whereas the positive ellipticity at 241 nm results primarily from one or more exposed tyrosines. Clearly, there is a large reduction in the stability of the protein in Gdn·HCl solutions upon removal of the four C-terminal residues. Significantly, the unfolding is completely reversible under these conditions. The slope of $[\theta]$ *vs.* Gdn·HCl concentration in the pretransition region differs in the two proteins. This probably indicates a slightly different environment of one or more of the exposed tyrosyl groups since the disulfide bridges are fairly inaccessible, at least in RNase A.

Free Energy of Unfolding. Salahuddin and Tanford (1970) have shown that the unfolding of RNase A in Gdn·HCl is a two-state process. We have found for RNase P that the various spectral properties, which are indicative of different portions of the protein, yield the same degree of unfolding at a given Gdn·HCl concentration. This is a necessary condition for the two-state mechanism (Lumry *et al.*, 1966; Hermans and Acampora, 1967). Another discriminating test for two-state transitions involves kinetic studies of unfolding and refolding in the transition region (Tanford, 1968, 1970; Aune and Tanford, 1969a). Figure 7 shows some typical kinetic plots obtained from difference absorption measurements at 287 nm. The two-state transition theory requires such plots to be

[3] We have also resolved the far-ultraviolet CD spectrum of a carbohydrate derivative of RNase A which contains 1 mole of glucosamine and 2–3 moles of hexose/mole of protein (D. Puett, unpublished results). The spectrum appears identical with that of RNase A and the resolved bands are located at 219, 206, and 195 nm with respective rotational strengths of -4.69×10^{-40}, -4.20×10^{-40}, and $+4.52 \times 10^{-40}$ cgs unit.

linear, and moreover, the value of $\Delta\epsilon$ extrapolated to zero time must agree with the equilibrium value. Also, the slope of the kinetic plots, the negative of which is equal to the sum of the rate constants for unfolding and refolding, must be the same at the transition midpoint whether one approaches this from the native or from the unfolded conformation. These various criteria were met for RNase A (Salahuddin and Tanford, 1970; D. Puett, unpublished results) and we find the same to hold for RNase P. These results, in conjunction with the equilibrium data, convincingly demonstrate the validity of the two-state transition model for RNase P.

Thus, the data in Figure 6 can be analyzed to yield the equilibrium constant, K, for the forward reaction in the equilibrium

$$N \rightleftharpoons U \tag{3}$$

where N refers to the globular conformation existing at neutral pH in dilute buffers and U denotes the unfolded, cross-linked protein in concentrated Gdn·HCl. The degree of the reaction is given by

$$\zeta = \frac{[\theta]_N - [\theta]}{[\theta]_N - [\theta]_U} \tag{4}$$

where $[\theta]$ represents the ellipticity at a given Gdn·HCl concentration and $[\theta]_N$ and $[\theta]_U$ represent the ellipticity of the native and unfolded protein *extrapolated* to the same concentration of Gdn·HCl. In the two-state process, the equilibrium constant is readily obtained as the ratio, $\zeta/(1 - \zeta)$, from which the free energy can be determined. Using this procedure and the data in Figure 6, as well as difference spectral data, the free energy of unfolding of both proteins in Gdn·HCl was determined and the results are given in Figure 8. As mentioned earlier, it can be seen that essentially the same free energy is obtained whether one uses the ellipticity (at either 275 nm or at 241 nm) or the ultraviolet difference data.

From the solubility of amino acids and either dipeptides or tripeptides in water and in urea or Gdn·HCl solutions, it is possible to calculate the free energy of transfer of the various side chains and peptide units from water to the particular denaturant concentration of interest (Tanford, 1964; Hermans *et al.*, 1969). The following relation enables one to fit unfolding data as a function of denaturant concentration (Tanford, 1964, 1970).

$$\Delta G = \Delta G_0 + \alpha \sum_i \Delta G_i \tag{5}$$

where ΔG_0 is the free energy of unfolding in the absence of Gdn·HCl and thus represents the intrinsic free energy of stability of the native protein relative to the unfolded protein; α denotes an average degree of exposure of the side chains and the peptide groups. The solid lines in Figure 8 were obtained using eq 5 in conjunction with the solubility data of Robinson and Jencks (1965) and of Nozaki and Tanford (1970), making certain assumptions regarding various residues.[4] The parameters we obtained are given in Table III.

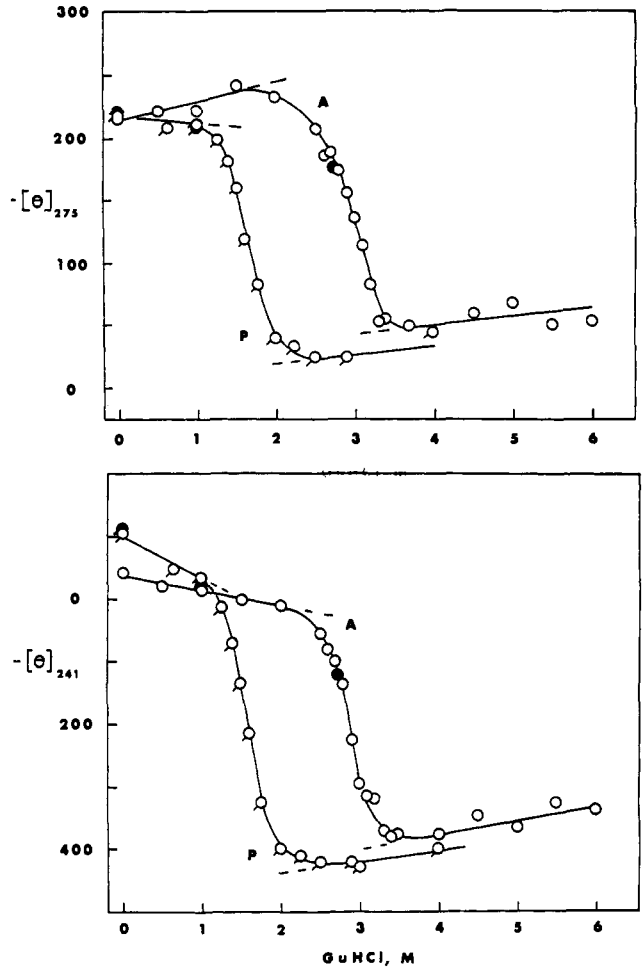

FIGURE 6: The variation of the molar ellipticity at 275 nm (upper) and at 241 nm (lower) for RNase P (○) and RNase A (○) with guanidine hydrochloride concentration. Each point represents a separate solution; open circles denote solutions prepared from protein stock solutions in water and closed circles refer to solutions prepared from protein originally in concentrated guanidine hydrochloride. The conditions are the same as those in Figure 2.

For RNase A, Salahuddin (1968; referred to by Tanford, 1970) reported $\alpha = 0.35$, and $\Delta G_0 = 10.5$ kcal/mole; the increased value we find for ΔG_0 may reflect the presence of phosphate in our system, and possibly a different method using in obtaining the terms in the summation of ΔG_i in eq 5.

Significantly, we find that the same value of α, 0.30, will describe the slope of the free-energy-denaturant concentration curve for both RNases A and P. Whereas minor changes in tertiary structure cannot be detected by this method, the data strongly indicate that the derivative does not have a partially open, or flexible, structure.

[4] The reported transfer free energies (Nozaki and Tanford, 1970) of the side chains Ala, Asn, Gln, His, Leu, Met, Phe, Thr, Trp, and Tyr (determined from solubility measurements relative to Gly) were plotted at 1, 2, 4, and 6 M Gdn·HCl, and the values at any Gdn·HCl concentration were obtained from a smooth curve through the experimental points. Transfer free energies for the other residues were arrived at by either adding or subtracting the hypothetical solubility of methylene units, calculated as one-third the difference in the solubility of Leu and Ala. The charged groups of Lys, Arg, Glu, and Asp were assumed to have a zero transfer free energy, although corrections were made for the aliphatic portion of the side chain, *i.e.*, the number of methylene units. For the other side chains, we assumed: Ile = Leu; Ser = Thr-Ala; $^1/_2$-Cys = Met-Ala-CH_2; Pro = 3 × CH_2. Values for the peptide unit were taken from the data of Robinson and Jencks (1965) on *N*-acetyltetraglycine ethyl ester; the solubility, divided by four, was in good agreement with the data of Nozaki and Tanford (1970) on diglycine, glycine.

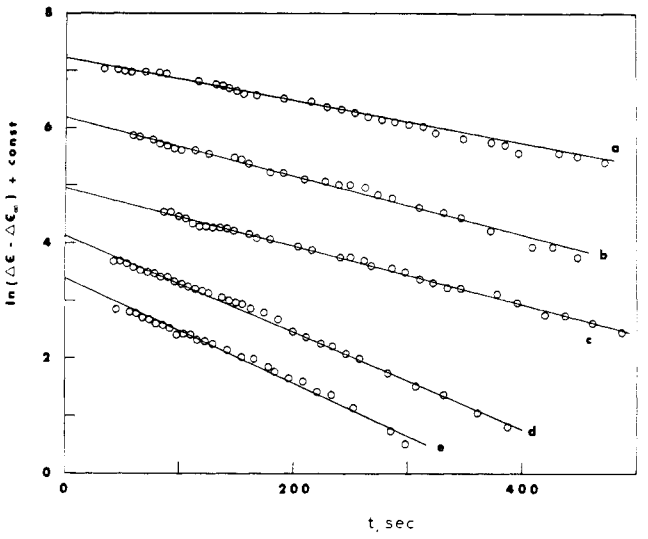

FIGURE 7: Kinetic plots of RNase P unfolding (a) and refolding (b–e), obtained from difference adsorption measurements at 287.5 nm. (a) 0–1.7 M, (b) 3.0–1.7 M, (c) 3.0–1.6 M, (d) 3.0–1.5 M, and (e) 3.0–0.83 M. The molarity (M) indicates guanidine hydrochloride; in addition all solutions contained 10 mM sodium phosphate. (To facilitate comparison of the various kinetic plots, the ordinate of each has been shifted by an arbitrary constant; this, of course, does not affect the slope.)

If one assumes that unfolding is due to ion binding, the generalized theory of linked functions (Wyman, 1964) can be simplified to give the expression (Tanford, 1970),

$$\Delta G = \Delta G_0 - RT \ln (1 + k a_{\pm})^{\Delta n} \qquad (6)$$

where k denotes the binding constant of the salt to the protein, $a_{\pm}$ is the mean ion activity of the salt which is equal to the square root of the salt activity, and Δn reflects the difference in the number of binding sites in the unfolded protein and the native protein. Using the data of E. P. K. Hade (reported by Aune and Tanford, 1969b) to convert Gdn·HCl molar concentration to activity, we find the values given in Table III. A unique set of parameters cannot be obtained, primarily because the data points are restricted to such a narrow range of Gdn·HCl concentration. Nevertheless, it can be seen that the ΔG_0 values resulting from this analysis are in agreement with the corresponding values using eq 5, and with the value of 13.0 reported by Salahuddin and Tanford (1970) for RNase A using this extrapolation method.

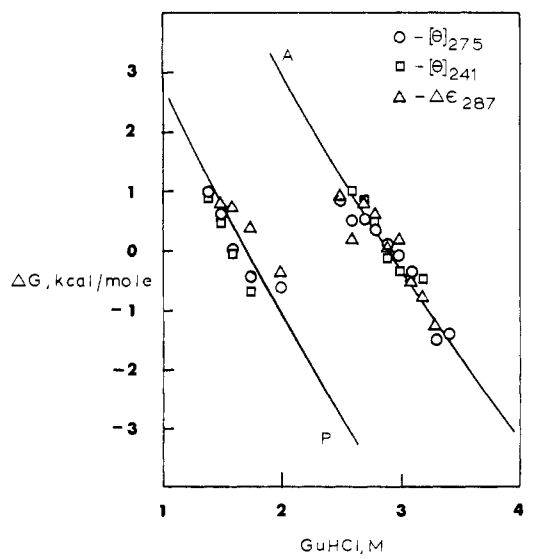

FIGURE 8: Dependence of the conformational free energy of RNase P (left) and RNase A (right) on the concentration of guanidine hydrochloride. As indicated in the figure, free-energy values were obtained from circular dichroic measurements at both 275 and 241 nm and from difference absorption spectral measurements at 287 nm. The lines are calculated using eq 5 and 6 and the parameters given in Table III.

TABLE III: Conformational Free-Energy and Unfolding Parameters of RNases P and A in Guanidine Hydrochloride and 10 mM Phosphate.

	RNase P	RNase A
ΔG_0 (kcal/mole)[a]	10.1	14.3
α	0.30	0.30
ΔG_0 (kcal/mole)[b]	9.5	14.2
k	1.0	1.0
Δn	28	30
ΔG_0 (kcal/mole)[b]	10.8	14.7
k	1.3	1.3
Δn	26	26
Average ΔG_0 (kcal/mole)	10.1 ± 0.7	14.4 ± 0.3

[a] Obtained using eq 5. [b] Obtained using eq 6. These parameters (*i.e.*, k, Δn, and ΔG_0) are not unique; the different sets shown give equally good fit to the data.

Effect of Temperature. Figure 9 shows the ellipticity of RNase P at 241 nm and at 275 nm as a function of temperature between 10 and 50°, and it can be seen that some thermal unfolding begins between 35 and 40°. Lin (1970) reported a transition temperature of 44.5° for RNase P. Whereas we did not reach an end point for the unfolded protein, it is clear that the midpoint of the transition is above 44.5°. This probably is a reflection of the stabilizing effect of the phosphate ion. Simons *et al.* (1969) found that below the thermal transition region of RNase A the ellipticity at 241 nm is very temperature dependent whereas the ellipticity at 275 nm is not. Between 20 and 40° the magnitude of the slope of $[\theta]_{241}$ *vs.* temperature for RNase A (Simons *et al.*, 1969) is about twice the value we find for RNase P. In contrast, we find that $[\theta]_{275}$ is slightly temperature dependent for RNase P below the transition region, but this is not the case with RNase A (Simons *et al.*, 1969).

Discussion

Evidence for Similarity in the Conformation of RNases A and P. The hydrodynamic data of Anfinsen (1956) indicate that RNase P has a stable folded conformation. The spectral studies reported herein show that this conformation is, in fact, very similar to that of RNase A. The near ultraviolet CD spectrum of the two proteins is almost identical, thus indicating that the local environment of the tyrosyl groups and the disulfide bridges has not been appreciably altered upon removal of the four C-terminal residues. However, the changes in the ellipticity with either Gdn·HCl or temperature in the pretransition region suggests that small environmental differences exist, probably involving one or more

exposed tyrosyl groups. The denaturation ultraviolet difference spectrum of the two proteins shows that the same number of tyrosyl groups (*ca.* 2–3) are inaccessible to water in the native or pseudo-native state.

The far-ultraviolet CD spectrum of RNase A is altered somewhat upon removal of the C-terminal tetrapeptide. This reflects a slightly different environment, and perhaps a slightly different conformation of some of the peptide chromophores. However, it is not unlikely that certain side chains contribute in this region as well, and thus some of the differences in the CD spectra of RNases A and P may be due to one or more side chains and not just to the peptide groups. Unfortunately, the complicated nature of the far ultraviolet CD spectrum, arising mainly from overlapping conformation-dependent bands, prohibits a quantitative interpretation of the resolved bands. The rotational strength of the 218 nm band in RNase P does suggest, however, that most of the ordered secondary structure is conserved in the derivative.

Conformational Free Energy of RNases A and P. Our results clearly show the importance of the C-terminal region in stabilizing the tertiary structure of RNase A. In a buffer containing 10 mM sodium phosphate and 0.1 M KCl, we find a ΔG_0 of 14.4 ± 0.3 kcal/mole for RNase A, whereas ΔG_0 of RNase-P is only 10.1 ± 0.7 kcal/mole. Thus, the removal of four residues (3.2% of the total residues) from the C terminus reduces the conformational free energy by 30 ± 9%. Even a linear extrapolation of these ΔG_0 values to shorter chain lengths leads to the expectation that derivatives with 7–12 fewer C-terminal residues than RNase P would be unstable,[5] *i.e.*, $\Delta G_0 < 0$. These results strongly suggest that stable chain folding occurs only when most of the protein is biosynthesized.

Rationalization of the Reduced Stability of RNase P. Based on the known crystal structure of ribonuclease (Kartha *et al.*, 1967; Wyckoff *et al.*, 1970; Richards and Wyckoff, 1971), one would like to calculate the *difference* in the conformational stability of RNases A and P resulting from interactions involving Asp-121, Ala-122, Ser-123, and Val-124 with the remainder of the protein. Although several factors prohibit an exact calculation of the free energy resulting from these interactions, it is possible to make certain estimates.

The conformational entropy of RNase P in concentrated Gdn·HCl must be somewhat lower than that of RNase A in the same solvent. This is due to the reduction in the number of residues in RNase P which consequently reduces the number of rotational states of the polypeptide backbone, and probably the side chains as well. Based on the calculations of Brant *et al.* (1967) one would expect the conformational entropy of the backbone of simple homopolypeptides to be about 10 eu for each residue, and a few more for long side chains. However, as discussed by Tanford (1970), these values probably overestimate the contribution of the conformational entropy in proteins; clearly, they are not applicable to the protein portion containing disulfide bonds. Certainly, a more reasonable value is that of 3–5 eu/residue[6] (Schellman, 1955b; Schachman, 1963). These values would predict a stabilization of about 3.6–6.0 kcal/mole in RNase P. Also, one can use the free energy value (0.9 kcal/mole per bond) given by Laskowski and Scheraga (1954), in the manner suggested by Hermans (1966), to correct for the freezing of rotations about the polypeptide main-chain valence bonds.[6] Assuming only the ϕ,ψ dihedral angles of the polypeptide backbone are affected (Hermans, 1966), this factor would lead to a stabilization of 7.2 kcal/mole in RNase P. Thus, these rough estimates of the conformational entropy yield a stabilizing free energy of about 5.4 ± 1.8 kcal/mole in RNase P.

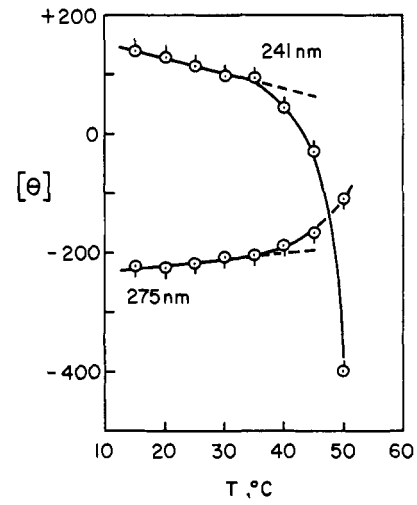

FIGURE 9: The effect of temperature on the molar ellipticity of RNase P at 275 nm (○) and at 241 nm (◌). The protein concentration was 0.7 mg/ml and the solution contained 10 mM sodium phosphate and 0.1 M KCl.

The loss of favorable interactions due to removal of the C-terminal tetrapeptide from RNase A can be estimated using model compound data for the free energy of hydrogen-bond formation (Schellman, 1955a; Hermans, 1966; Poland and Scheraga, 1965) and the transfer free energy of side chains and peptide groups from organic solvents to water (Nozaki and Tanford, 1971). Recent theoretical treatments also enable one to estimate the free energy associated with the formation of hydrophobic bonds (Nemethy and Scheraga, 1962; Nemethy, 1967). We have examined a Kendrew skeletal model[7] of RNase S, and the various interactions that the four C-terminal residues can have with the remainder of the protein are summarized in Table IV. Using reasonable values for the various stabilizing interactions in RNase A we estimate that RNase P should be about 7.3 ± 0.9 kcal less stable than RNase A.

Considering both the loss of stabilizing interactions and the conformational entropy, one would expect RNase P to be roughly 1.9 ± 2.7 kcal less stable than RNase A. Although the calculated value is in reasonable agreement with the experimental value of 4.3 ± 1 kcal, some difference between the predicted and observed conformational free-energy difference is anticipated. This would result both from errors involved in applying model compound data to real proteins, as well as from failure to account for the loss of other stabilizing interactions in the modified protein. The latter probably

[5] Some CD measurements on des-(120–124)-RNase and des-(119–124)-RNase suggest that these derivatives have a stable folded structure. They are, however, unfolded in the presence of low Gdn·HCl concentrations, and their ΔG_0 is estimated to be quite low (D. Puett, unpublished results).

[6] Strictly speaking, this value(s) is not applicable to the present situation and its use implies that RNase P behaves as a protein of 124 residues with complete rotational freedom about the ϕ,ψ angles of the four C-terminal peptide linkages, whereas in RNase A these bonds are completely frozen.

[7] It is a pleasure to acknowledge Susan B. Puett for construction of the model; the coordinates were those of Wyckoff *et al.* (1970).

TABLE IV: Interactions of the Four C-Terminal Residues of RNase A with the Remainder of the Protein.[a]

Interaction	Estimated Energy[b] (kcal)
A. Peptide–peptide hydrogen bonds[c]	
1. NH of Ser-123 with CO of His-105	1.5
2. CO of Ala-122 with NH of Ile-106	1.5
3. NH of Phe-120 with CO of Ile-107	1.5
B. Salt linkages[d]	
4. α-COO^- of Val-124 with ϵ-NH_3^+ of Lys-104	1.0
C. Hydrophobic interactions[e]	
5. Val-124-γ_1-CH_3 and Ala-122-β-CH_3 with Ile-107-δ_1-CH_3	0.6
6. Val-124-γ_2-CH_3 and His-105-β-CH_2	0.3
D. Free energy of transfer of the C-terminal tetrapeptide from an organic solvent to water assuming 30–50% exposure in the native state[f]	1.7–2.7
Total estimated reduction in free energy due to removal of the C-terminal tetrapeptide (*i.e.*, A + B + C or A + B + D above)	6.4–8.2

[a] Based on a Kendrew skeletal model of RNase S. [b] The given energies refer to the loss in energy due to removal of the particular groups. [c] The free energy of hydrogen bond disruption is from Schellman (1955a). [d] This value is only a crude approximation since the dielectric constant is not known precisely. [e] Estimated from the calculations of Nemethy and Scheraga (1962) and Nemethy (1967). [f] Calculated from the solubility data of Nozaki and Tanford (1971); the transfer free energy refers to both the side chains and the peptide groups.

involves Phe-120, His-119, and perhaps other residues near the C terminus, and of course the active site, as well. In fact, it is almost essential to invoke such changes to account for reduction in the enzymic activity. One possible driving force for a change in position of Phe-120 may result from the formation of the new α-carboxyl group in a partially hydrophobic environment, or even perhaps an unfavorable electrostatic interaction between the α-carboxyl group of the derivative and the phosphate ion. In either case, rotation about the ϕ,ψ bonds of His-119 could place the ionized carboxyl group in better contact with water, and also increase the distance between the phosphate ion and the α-carboxyl group.

Implications for in Vivo Protein Folding. With RNase A it is well established that the completely unfolded and reduced protein can refold to give a biologically active structure under *in vitro* conditions (Anfinsen and Haber, 1961). Pflumm and Beychok (1969) have shown that if corrections are made for the fraction of reoxidized RNase A that is enzymically inactive, *i.e.*, irreversibly denatured, the CD spectrum of reoxidized RNase A is identical with that of native RNase A, except near 240 nm where the intensity of the reoxidized protein is somewhat less than that of the native protein. Reversible unfolding is a necessary condition for the biologically active conformation to correspond to that of the lowest free energy.

Since Taniuchi (1970) found that reduced, unfolded RNase P could not spontaneously refold to the pseudo-native structure, it is tempting to speculate that under *in vivo* conditions folding of the nascent polypeptide chain does not occur until after Phe-120 has been added. We have, however, shown that RNase P can be *reversibly* unfolded with the disulfide bonds intact. Thus, under these conditions it is concluded that the polypeptide chain containing residues 1–120 does have sufficient information to return to the folded conformation that is almost identical to RNase A, although the conformational stability of the derivative is greatly reduced. Evidently the reduction in entropy due to the intact disulfide bonds of unfolded RNase P is sufficient to permit the modified protein to refold. It is possible that a similar situation occurs during protein biosynthesis, since attachment of the nascent polypeptide chain to the ribosome undoubtedly reduces the entropy of the incompleted protein. Thus, as our results suggest, it is possible that stable folding is initiated just before Phe-120 is added.

In any case, these results, as well as those of Taniuchi (1970), indicate that the folded structure of RNase A is stable only when most of the polypeptide chain has been biosynthesized. This is in agreement with our conclusions on apomyoglobin (Hermans and Puett, 1971; Puett, 1972), but does not support the hypothesis that a stable tertiary structure develops during the early stages of biosynthesis (De Coen, 1970). This raises interesting questions regarding the development of a stable tertiary structure in proteins during evolution, and shows the importance of both the chemical nature and size of the primordial gene for a particular protein.

Relationship of These Results to Studies on Other Proteins. In the case of apomyoglobin, studies on various fragments have indicated that tertiary structure begins to form only after the polypeptide chain is at least 50% synthesized (Epand and Scheraga, 1968; Atassi and Singhal, 1970; Singhal and Atassi, 1970; Hermans and Puett, 1971; Puett, 1972). These results are consistent with the two-state model for unfolding (Lumry *et al.*, 1966) since they suggest that stable intermediates do not occur. In addition, Anfinsen and coworkers (*cf.* the review by Anfinsen *et al.*, 1971) have shown that staphylococcal nuclease, which like apomyoglobin contains no disulfide bridges, must have essentially all of the amino acid residues to obtain a stable tertiary structure and biological activity. Hartley (1970) has also shown that removal of a few C-terminal residues inactivates barnase.

Although there have been studies on the S-peptide (Tamburro *et al.*, 1968b; Klee, 1968; Brown and Klee, 1969, 1971) and S-protein of RNase S (Sherwood and Potts, 1965; Simons and Blout, 1968; Simons *et al.*, 1969), nothing has been done on N-terminal fragments comprising *ca.* 50% of RNase A. However, in view of our results and those of Taniuchi (1970), one would expect little or no stable tertiary structure to be formed in N-terminal fragments of RNase A containing 60–70 residues. The removal of Phe-120, or of both Phe-120 and His-119, from RNase P causes complete inactivation (Lin, 1970), although activity can be regained in both derivatives by adding a peptide corresponding to the 14 C-terminal residues of RNase A (Lin *et al.*, 1970).

In summary, we have demonstrated that removal of the four C-terminal residues from RNase A reduces the conformational free energy by some 30%. This indicates that a significant amount of stable tertiary structure occurs only during the terminal stages of biosynthesis. Lastly, we wish to point out that our results are consistent with the two-state model for intact proteins, since the data suggest that

even the derivative, RNase P, changes conformation *via* an equilibrium two-state mechanism.

Acknowledgments

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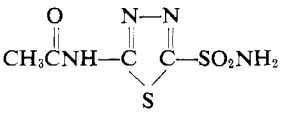
CORRECTIONS

"Studies of the Histidine Residues of Carbonic Anhydrases Using High-Field Proton Magnetic Resonance," by Jack S. Cohen, Chung T. Yim, Marianne Kandel, Allan G. Gornall, Stephen I. Kandel, and Murray H. Freedman, Volume 11, Number 3, February 1, 1972, page 327.

On page 331, the structure of Diamox is incorrect. The correct structure should be



"Conversion of Retinyl Methyl Ether into Retinol in the Rat *in Vitro*," by S. Narindrasorasak and M. R. Lakshmanan, Volume 11, Number 3, February 1, 1972, page 380.

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