Chain Folding in Ribonuclease A Derivatives Lacking Five and Six Carboxyl-Terminal Residues†

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ABSTRACT: The peptic derivative of bovine pancreatice ribonuclease (RNase A), *i.e.*, RNase P or des-(121–124)-RNase, has been reacted with carboxypeptidase A under different conditions to obtain the enzymically inactive derivatives, des-(120–124)-RNase and des-(119–124)-RNase. The circular dichroic (CD) spectrum of each derivative was obtained and the magnitude of the molar ellipticity was found to be somewhat less than that of RNases A and P at all wavelengths between 200 and 300 nm. The resolved major tyrosine (276–278 nm) and disulfide (269 nm) CD bands were present, although the rotational strength of each was found to be less than that of RNases A and P. Above 200 nm, the major difference in the CD spectrum of these derivatives and that of RNase P is in the 241-nm region; this indicates an altered environment of one or more tyrosyl groups upon removal of Phe-120. Denaturation difference spectroscopy of des-(119–124)-RNase indicated that *ca.* two tyrosyl groups are inaccessible to water under nondenaturing conditions, thus suggesting that this derivative has a stable folded or partially folded structure. The derivatives were found to have a much lower conformational stability than RNase P as estimated from the reversible unfolding in guanidine hydrochloride. These results establish the importance of the carboxyl-terminal region, and in particular of the hydrophobic residue Phe-120, in the development of a stable tertiary structure in RNase A.

It is known that the tertiary structure of the peptic derivative of RNase A,[1] *i.e.*, des-(121–124)-RNase or RNase P, is quite similar to that of the intact protein (Anfinsen, 1956; Sela and Anfinsen, 1957; Taniuchi, 1970; Puett, 1972a), although the enzymic activity of the derivative is only a few per cent of that corresponding to RNase A (Lin, 1970). We have recently shown that the conformational free energy of unfolding of RNase P is about 30% lower than that of the intact protein (Puett, 1972a). Even a linear extrapolation of these ΔG_0 values to shorter chain lengths suggested that derivatives lacking *ca.* 10 more carboxyl-terminal residues than RNase P would be unstable. The implication of these results to *in vivo* chain folding is that the carboxyl-terminal region of RNase A is of crucial importance in stable chain folding. Similar conclusions have been made for apomyoglobin (Atassi and Singhal, 1970; Hermans and Puett, 1971; Puett, 1972b) and staphylococcal nuclease (Anfinsen *et al.*, 1971). These data indicate that a stable tertiary structure is formed only during the terminal stages of biosynthesis, and not during the early stages as hypothesized (De Coen, 1970).

In this paper, both near- and far-ultraviolet circular dichroic spectra are presented for two RNase A derivatives lacking five and six carboxyl-terminal residues, *i.e.*, des-(120–124)-RNase and des-(119–124)-RNase, respectively; also, denaturation difference (absorption) spectra are given for the latter. Such studies are useful in comparing the secondary and tertiary structure of the dervivatives to that of RNases A and P. In order to estimate the conformational stability of the derivatives relative to RNases A and P (Puett, 1972a), the effect of guanidine hydrochloride on the spectral properties was also investigated. Lin (1970) has shown from absorbance measurements at 287 nm that these derivatives are devoid of enzymic activity and have thermal transition temperatures lower than both RNases A and P. These results clearly demonstrate a reduced stability of the various derivatives relative to RNase A. However, from these studies alone it is difficult to assess the nature of the folded, or partially folded, conformation of the derivatives with regard to the known conformation of the intact protein (*cf.* the review by Richards and Wyckoff, 1971).

Experimental Section

RNase P was prepared from RNase A (Sigma XII-A) and purified as described elsewhere (Puett, 1972a). Carboxypeptidase A (Worthington DFP treated) was used as described by Lin (1970) to selectively remove just Phe-120 or both Phe-120 and His-119 from RNase P.

Typically, 20 mg of RNase P was incubated at 45° for 10 min with carboxypeptidase A (0.2–0.4 mg to prepare des-(119–124)-RNase and 60 μg to prepare des-(120–124)-RNase). Norleucine was present as an internal standard in the incubation mixture in the same molar concentration as RNase P. The digest was applied directly to a 1.5 × 54 cm column of Bio-Gel P-10, equilibrated and developed with 5% acetic acid at 25°, and the effluent was monitored at 280 nm. The protein peak (*ca.* 50 ml) was pooled, minus the first few tubes that contained the carboxypeptidase, and lyophilized. To collect the free amino acids, the effluent volume from about 60 to 140 ml was pooled and lyophilized; calibration of the column with RNase A and various amino acids ensured that

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[1] Abbreviations used are: RNase A, the major bovine pancreatic ribonuclease; RNase P, the peptic derivative of RNase A, *i.e.*, des-(121–124)-RNase; RNase S, the subtilisin derivative of RNase A; Gdn·HCl, guanidine hydrochloride; ΔG_0, the conformational free energy of unfolding at neutral pH in the absence of denaturant.

these conditions gave a clear separation of the protein from the amino acids. Citrate buffer was added to the lyophilizing flask containing amino acids and these were analyzed directly on the Beckman 120 amino acid analyzer. The recovery of norleucine was invariably greater than 90%.

The removal of both Phe-120 and His-119 was quantitative, and thus the desalted, lyophilized protein from the Bio-Gel P-10 column was ready for use. Some difficulty was experienced, however, in preparing des-(120–124)-RNase. Our optimum conditions (pH 5.0, see Lin, 1970) yielded a mixture of 70% des-(120–124)-RNase and 30% des-(119–124)-RNase. These were separated using two cycles of ion-exchange chromatography (SP-Sephadex C-25, 0.13 M sodium phosphate (pH 6.45), 0.9 × 13 cm column), followed by desalting on a Bio-Gel P-4 column (2 × 58 cm) equilibrated with 5% acetic acid, with lyophilization of the major protein pool after each chromatographic run.

Circular dichroic (CD) measurements were made on the Cary 60 spectropolarimeter with the Model 6002 CD attachment. The experimental conditions and the methods used in curve resolution were the same as described earlier (Puett, 1972a). Denaturation difference spectra were obtained with the Hitachi Perkin-Elmer EPS-3T double-beam recording spectrophotometer in the 90–110% or the 50–150% T mode.

Protein stock solutions were prepared by dissolving a weighed amount of the lyophilized protein in distilled water. The actual protein concentrations were, however, determined from quantitative amino acid analysis (6 N HCl, 110°, 20 hr) on an aliquot from the stock solution, using norleucine as an internal standard (recovery was 85–90%). Due corrections were made for cysteine and for serine and threonine (5%). All protein solutions contained 10 mM sodium phosphate, and either 0.1 M KCl or Gdn·HCl (Heico spectrophotometric grade). The enzymic assay was based on the release of acid-soluble oligonucleotides from yeast RNA as previously described (Puett, 1972a).

Results

Amino acid analysis of each derivative confirmed the removal of Phe only in des-(120–124)-RNase and of both Phe and His in des-(119–124)-RNase.[2] The values for the other amino acids agreed within a few per cent of those for RNase P. As expected (Lin, 1970), the derivatives were found to be devoid of enzymic activity.

CD spectra of the derivatives are presented in Figure 1 and the rotational strengths of the resolved major gaussian bands are given in Table I. The near-ultraviolet CD spectrum of RNase A is due primarily to exposed tyrosines and disulfides, with minor contributions from inaccessible tyrosines and from phenylalanyl residues (Horwitz *et al.*, 1970; Puett, 1972a). The molar ellipticity of both RNases A and P at 275 nm is about −217 (deg cm²)/dmole (Puett, 1972a). The corresponding values for des-(120–124)-RNase and des-(119–124)-RNase are −180 and −168, respectively, corresponding to reductions of 17 and 22%. The lower ellipticity values are also reflected in the reduced rotational strengths

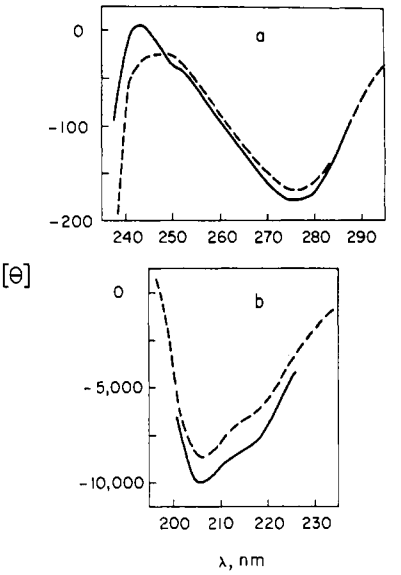

FIGURE 1: The near- (a) and far- (b) ultraviolet CD spectrum of des-(120–124)-RNase (———) and of des-(119–124)-RNase (– – – –) in 0.1 M KCl–10 mM sodium phosphate (pH 7) at 26°. The protein concentration was *ca.* 0.5 mg/ml above 235 nm (1-cm path length) and *ca.* 0.1 mg/ml below 235 nm (0.5- and 1-mm path length).

of both the major resolved tyrosyl (276–278 nm) and disulfide (269 nm) bands.

At wavelengths near 240 nm, the CD spectrum of the derivatives is different from that of RNases A and P. The positive band in RNase A is due primarily to tyrosine (*cf.* Timasheff, 1970) with perhaps small contributions from phenylalanine (Puett, 1972a). The rotational strength of the positive 235-nm resolved band of these derivatives is less than the corresponding values for RNases A and P (Puett, 1972a).

Between 200 and 240 nm, the CD spectrum of des-(119–124)-RNase and of des-(120–124)-RNase is similar to the spectrum of RNase P, but below 200 nm the ellipticity of des-(119–124)-RNase seems lower, *i.e.*, less positive, than that of RNase P. There is considerable overlap of the conformation-dependent n–π^* and π–π^* bands in this region, and moreover, the n–π^* band of RNase A is blue shifted

TABLE I: Rotational Strengths (in 10^{-42} cgs unit) of the Resolved Major near-Ultraviolet CD Bands of RNase A and Derivatives.[a]

Protein	276–278 nm	269 nm	235 nm
RNase A[b]	−6.1	−13.1	+12.0
RNase P[b]	−6.0	−12.4	+16.9
Des-(120–124)-RNase	−4.5	−10.7	+9.1
Des-(119–124)-RNase	−4.5	−9.7	+4.1

[a] Both the 276- to 278-nm band and the 235-nm band are assigned to tyrosine; the 269-nm band is assigned to disulfides (Horwitz *et al.*, 1970; Timasheff, 1971; Puett, 1972a). The minor CD bands have not been included in this table since they are subject to appreciable error unless a large number of scans are available. [b] These results are from the earlier paper (Puett, 1972a).

[2] Normalized to 10 moles of lysine/mole of protein, we found des-(120–124)-RNase to contain 3.7 moles of histidine, which is in good agreement with the value obtained under similar hydrolysis conditions for RNase A (Hirs *et al.*, 1954), and 1.8 moles of phenylalanine (expected value is 2.0); des-(119–124)-RNase contained 3.0 and 2.1 moles of histidine and phenylalanine, respectively, in excellent agreement with the expected values of 3.0 and 2.0. It is a pleasure to thank Mr. John D. Ford for performing the analyses.

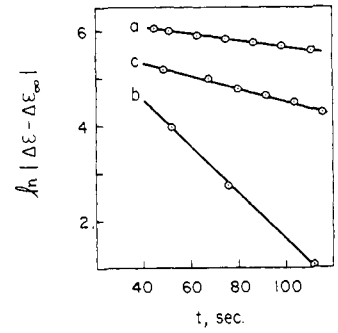

FIGURE 2: Kinetic plots of des-(119–124)-RNase unfolding: (a) 0–0.75 M Gdn·HCl, (b) 0–4 M Gdn·HCl, and refolding (c) 1.5–0.75 M Gdn·HCl, as obtained from difference absorption measurements at 287 nm.

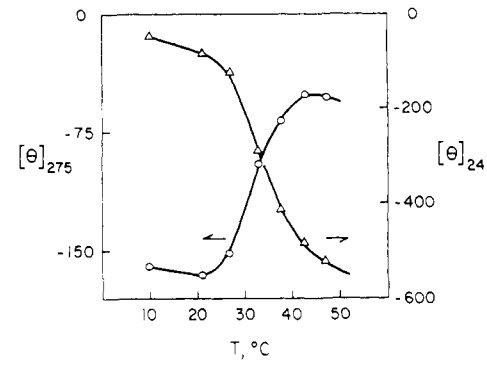

FIGURE 4: The effect of temperature on the molar ellipticity of des-(119–124)-RNase in 0.1 M KCl and 10 mM sodium phosphate.

relative to the normal α-helical CD n–π* band (Schellman and Lowe, 1968). Also, the aromatic groups may contribute to the optical activity in this region. Thus, it is difficult to assess precisely the nature of the secondary structure from far-ultraviolet CD measurements alone. It seems obvious, however, that much of the secondary structure in RNase A is preserved in these derivatives. In fact, the rotational strength of the n–π* band in the derivatives (centered at 215.5 nm) is between -4.5×10^{-40} and -5.4×10^{-40} cgs unit, which is within experimental error of the value found for RNase P (Puett, 1972a), although in the latter derivative the band was located at 217.5 nm.

The denaturation difference spectrum of des-(119–124)-RNase has the general shape as that found for RNase P (Puett, 1972a), although $\Delta\epsilon$ at the 287 nm extremum is somewhat less, *e.g.*, $-\Delta\epsilon_{287}$ for des-(119–124)-RNase in 0.75, 1.0, and 4.0 M Gdn·HCl is, respectively, 780, 1110, and 1390 absorbance units per (cm M). In 4 M Gdn·HCl the magnitude of $\Delta\epsilon$ is somewhat lower than the values of −1800 and −1600 for RNases A and P, respectively (Puett, 1972a), and is consistent with the exposure of *ca.* two tyrosyl groups (Donovan, 1969).

Using the 287-nm (difference) absorbance extremum to monitor conformation changes, the kinetics of unfolding and refolding in the transition region (0.75 M Gdn·HCl), as well as unfolding in 4 M Gdn·HCl, are shown in Figure 2 for des-(119–124)-RNase. The linearity of these plots is consistent with a two-state transition (Tanford, 1970), although more extensive data are required to determine if the transition is indeed two state.

The variation of the molar ellipticity of the two derivatives at several wavelengths with denaturant concentration is presented in Figure 3. The changes in the molar ellipticity at 275 and 241 nm reflect changes in tertiary structure, whereas the changes at 217.5 nm primarily denote changes in the ordered secondary structure. These transitions are completely reversible as judged by the return of the spectral properties upon dilution from concentrated Gdn·HCl solutions. Also, solutions prepared from samples in concentrated Gdn·HCl that had been desalted (either *via* gel filtration or ultrafiltration) and lyophilized gave almost identical spectra as that obtained before treatment with the denaturant. Similar plots for RNases A and P gave cooperative transitions with transition midpoints at 2.9 and 1.7 M Gdn·HCl, respectively (Puett, 1972a).

Figure 4 shows the effect of temperature on the molar ellipticity at both 275 and 241 nm of des-(119–124)-RNase. The transition midpoint is at about 32°, in excellent agreement with Lin's (1970) results obtained from absorbance measurements. Although phosphate stabilizes RNases A, S, and P (*cf.* Sela and Anfinsen, 1957; Sherwood and Potts, 1965; Lin, 1970; Puett, 1972a), evidently this is not the case with des-(119–124)-RNase. Presumably this reflects the absence of phosphate binding to des-(119–124)-RNase; in fact, Lin (1970) has shown that this derivative, as well as des-(120–124)-RNase, does not bind the inhibitor 2′-cytidylic acid.

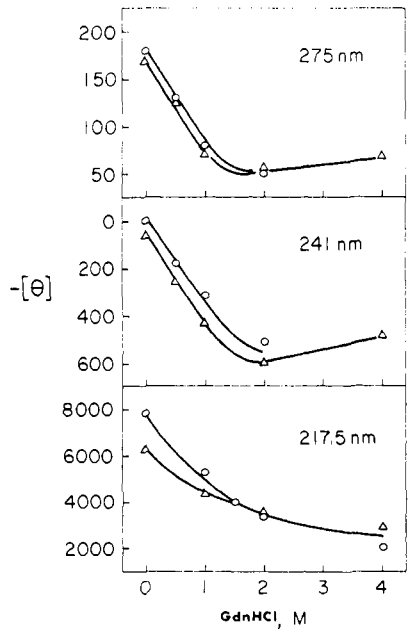

FIGURE 3: Dependence of the molar ellipticity at the indicated wavelengths on the concentration of Gdn·HCl for des-(120–124)-RNase (○) and des-(119–124)-RNase (△) at 26°. All solutions contained 10 mM sodium phosphate.

Discussion

The CD spectrum of des-(120–124)-RNase is quite similar to that of des-(119–124)-RNase, although the ellipticity of the latter is somewhat less at all wavelengths. The near ultraviolet CD spectrum of these derivatives is slightly different from that of RNase P, thus indicating a slightly altered environment of the tyrosyl groups (primarily those that are exposed) and of the disulfides.

The denaturation difference spectrum of des-(119–124)-RNase suggests that two tyrosines are inaccessible to water under nondenaturing conditions. This result strongly suggests that this derivative has a stable folded conformation[3] that may be similar to, but not necessarily identical with, that of RNase A which has more than two inaccessible tyrosines.

The folded structure in the derivatives is, however, easily disrupted by relatively low concentrations of Gdn·HCl, thus indicating a low free energy of unfolding. In fact, the equilibrium (Gdn·HCl induced) unfolding curve shown in Figure 3 can be interpreted in terms of a two-state transition (Lumry *et al.*, 1966) characterized by a low ΔG_0[4] at ambient temperature. Thus, the addition of even small amounts of Gdn·HCl would tend to lower the thermal transition midpoint below 26°, and the isothermal unfolding curve, *i.e.*, optical parameter *vs.* Gdn·HCl concentration, would not exhibit the characteristic "sharp" transition with two well-defined end points corresponding to the folded and the unfolded protein.

Since a stable end point was not found at low Gdn·HCl concentrations, one could argue that the stable folded structure is in equilibrium with an appreciable amount of the unfolded protein, and the transition may or may not be two state. However, this does not appear to be the case since the thermal unfolding profile, like that of RNase A (Hermans and Scheraga, 1961; Brandts and Hunt, 1967), exhibits two well-defined end points, thus suggesting that the folded structure is the predominant form at 26°. A multistate transition cannot be completely ruled out, but the linear kinetic plots of unfolding and refolding argue against this (*cf.* Tanford, 1970).

The major finding is, of course, that the stability of the folded conformation is appreciably reduced upon removal of Phe-120 or of Phe-120 and His-119 from RNase P. Part of this instability may arise from the formation of the α-carboxyl group (on His-119 or on Val-118) in a hydrophobic region in RNase P.

The data indicate that derivatives lacking a few more carboxyl-terminal residues than des-(119–124)-RNase would be unstable. An extrapolation of these findings to *in vivo* protein biosynthesis and chain folding is difficult, primarily because it is not yet possible to evaluate the entropy changes due to closure of the disulfide bridges and the entropy effects due to attachment of the nascent polypeptide chain to the ribosome. These results do, however, establish the importance of the carboxyl-terminal region in the development of a stable tertiary structure in a relatively small protein. It will be of interest to determine if the same relationships hold for both larger and smaller proteins, and for proteins exhibiting quaternary structure.

[3] Based on the similarity in the CD spectrum of des-(119–124)-RNase and des-(120–124)-RNase, one expects that the latter derivative also contains approximately two inaccessible tyrosines since the tertiary structure in these two derivatives seems to be similar. It is possible that several folded or partially folded conformations of the derivatives exist; however, if so, there must be a low-energy barrier separating the conformations since the kinetics of unfolding and refolding, as well as the thermal unfolding, are consistent with the two-state mechanism.

[4] If one assumes the CD data at 275 and 241 nm in Figure 3 can be described in terms of a two-state transition, where the optical parameter at 0 M Gdn·HCl represents the folded conformation and the line between 2 and 4 M Gdn·HCl denotes the unfolded conformation, application of eq 5 in the previous paper (Puett, 1972a) yields ΔG_0 values of +0.87 and +0.80 kcal per mole for the derivatives, des-(120–124)-RNase and des-(119–124)-RNase, respectively. The average difference in the degree of exposure (relative to the unfolded protein) was found to be about 0.05 for both derivatives. This indicates that either a partially unfolded conformation is present in the absence of denaturant such that many of the hydrophobic groups are exposed to water, or that unfolding proceeds with one or more stable intermediates in the pathway.

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