assistance of Gertrude Neuman.

**Registry No.** 2,3-DPG, 138-81-8; GSH, 70-18-8; Mn, 7439-96-5; dysprosium, 7429-91-6.

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# Characterization of the Histidine Proton Nuclear Magnetic Resonances of a Semisynthetic Ribonuclease†

Marilynn S. Doscher, Philip D. Martin, and Brian F. P. Edwards*

ABSTRACT: The proton magnetic resonance spectrum at 300 MHz of the histidine residues in a semisynthetic derivative of bovine pancreatic ribonuclease (RNase A) has been determined. The derivative RNase 1–118·111–124 was prepared by enzymically removing six residues from the COOH terminus of the protein (positions 119–124) and then complementing the inactive RNase 1–118 with a chemically synthesized peptide containing the COOH-terminal 14 residues of ribonuclease (RNase 111–124) [Lin, M. C., Gutte, B., Moore, S., & Merrifield, R. B. (1970) *J. Biol. Chem. 245*, 5169–5170]. Comparison of the line positions of the C(2)-$^1$H resonances of these residues and of their pH dependence with those reported by other workers has allowed assignment of the resonances to individual residues, as well as the determination of individual p$K$ values for histidine-12, histidine-105, and histidine-119. The assignment of histidine-119 was confirmed by the use of a selectively deuterated derivative. The titration behavior of all four histidine residues is indistinguishable from that observed by others for bovine pancreatic ribonuclease A. Partial dissociation of the noncovalent semisynthetic complex was evident at 30 °C, pH 4.0, 0.3 M NaCl; pertinent spectra were analyzed to provide an estimate of the association constant between the component chains under these conditions of $1.9 \times 10^3$ $M^{-1}$.

Successive digestion by pepsin and by carboxypeptidase A of bovine pancreatic RNase A[1] removes six residues from the COOH terminus of the molecule to provide a shortened chain, RNase 1–118, that is devoid of enzymatic activity (Lin, 1970). Complementation of this inactive segment with a chemically synthesized tetradecapeptide comprising the COOH terminus of RNase, viz., residues 111–124, results in the formation of

† From the Department of Biochemistry, Wayne State University School of Medicine, Detroit, Michigan 48201. *Received January 3, 1983; revised manuscript received May 4, 1983.* This work was supported in part by National Institutes of Health Research Grant GM 29791 and a Grant-in-Aid from Wayne State University.

[1] Abbreviations: DSS, sodium 3-(trimethylsilyl)-1-propanesulfonic acid; DCC, dicyclohexylcarbodiimide; RNase A, bovine pancreatic ribonuclease A; RNase 1–118, polypeptide composed of residues 1 through 118 of RNase; RNase 111–124, tetradecapeptide composed of residues 111 through 124 of RNase; RNase 1–118·111–124, noncovalent complex of RNase 1–118 and RNase 111–124; Boc, *tert*-butyloxycarbonyl; RNase S, noncovalent complex of residues 1 through 20 and 21 through 124 formed by limited subtilisin digestion of RNase.

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a semisynthetic, noncovalent complex exhibiting 98% of the enzymatic activity of RNase A (Lin et al., 1970; Gutte et al., 1972).

Several analogues of the complex have been synthesized to test postulated roles for phenylalanine-120 and serine-123 in the mechanism of action of the enzyme (Lin et al., 1972; Hodges & Merrifield, 1975). Roles for both residues in the binding of substrate had been proposed, primarily from the structure in the crystal of RNase S–inhibitor complexes (Wyckoff & Richards, 1971). When phenylalanine-120 was replaced by leucine, the activity against cytidine cyclic 2′,3′-phosphate of the resulting complex was 13% of that seen with the phenylalanine-containing structure (Lin et al., 1972). The Michaelis constants at pH 6.0 for this substrate were not significantly different for the two enzymes, indicating that reduced substrate binding was not responsible for the lowered activity. When alanine replaced phenylalanine-120, only 0.8% of the original activity remained (Hodges & Merrifield, 1974); in this case, no further delineation of kinetic parameters was reported. The function of serine-123 was examined by replacing it with alanine (Hodges & Merrifield, 1975). The modification eliminated any possibility of hydrogen-bond formation between this residue and a proton of the amino group at position 4 of the cytosine ring in cytidine cyclic 2′,3′-phosphate or the carbonyl oxygen at position 4 of the uracil ring in uridine cyclic 2′,3′-phosphate. The expectation that substrate binding might be sharply lowered in the alanine-123 derivative was not realized, however, as it had full activity toward the cytidine substrate and 25% activity toward the uridine substrate (the observed assay velocity in the latter case being the result of an increase in the Michaelis constant from 11 mM to 62 mM that is somewhat compensated by a slight increase in turnover rate).

The discrepancy between the predicted and observed properties of the phenylalanine-120 and serine-123 analogues suggested, among other possibilities, that the detailed structure of RNase S did not provide a suitable model for the consideration of structure–function relationships in RNase 1–118·111–124. Large single crystals of the complex of a quality suitable for X-ray diffraction analysis and isomorphous with forms W and R of ribonuclease S are now in hand (Sasaki et al., 1979a,b). Very recently, our crystallization procedure has been modified with the result that 80% of the reflections between 2.5 and 2.0 Å are now readily observed (Doscher et al., 1983). A restrained least-squares refinement (Hendrickson & Konnert, 1980) of the structure is currently being carried out.

We report here the initiation of a complementary line of investigation involving examination of the histidine C(2) proton magnetic resonance spectra of RNase 1–118·111–124. Extensive data exist concerning the histidine proton magnetic resonance spectra of RNase A and RNase S; among the parameters determined are the identity of the individual resonances of all four histidine C(2) protons in these molecules, the dependence of line position on pH for each resonance (with concomitant determination of apparent p$K$ in each case), and perturbation of spectral positions and apparent p$K$ values upon the binding of a number of substrate-like ligands [for reviews, see Markley (1975a) and Jardetzky & Roberts (1981)].

## Experimental Procedures

*Materials.* RNase A (RAF OLA, salt-free) and pepsin (lot PM36c675, 2700 units/mg) were purchased from Worthington. Carboxypeptidase A (lot 46c-8030, 57 units/mg) and its substrate, hippuryl-L-phenylalanine (Sigma H-6875, lot 32C-0740), were from Sigma.

Amino acid intermediates were all obtained from Bachem, Inc. They included the α-Boc derivatives of valine, *O*-benzylserine, alanine, β-benzylaspartate, phenylalanine, $N^{im}$-DNP-histidine, proline, *O*-benzyltyrosine, glycine, γ-benzylglutamate, and the *p*-nitrophenyl ester of asparagine.

Dicyclohexylcarbodiimide, Pierce Chemical Co., 4-(dimethylamino)pyridine, 99%, Aldrich Chemical Co., triethylamine, Eastman Organics, and trifluoroacetic acid, 99%, Pierce Chemical Co., were used without further purification. Pyridine, Baker AR, was distilled from ninhydrin (1 g/L) before use. Dichloromethane, certified ACS, Fisher, was stored over anhydrous calcium chloride before use. *N*,*N*′-Dimethylformamide, certified ACS, Fisher, was stored over type 4A molecular sieves (Davison Chemical) before use. All other reagents and solvents were the highest grade available and were used without further purification.

*Preparation of RNase 1–118.* RNase 1–118 was prepared by successive digestion of RNase A with pepsin and carboxypeptidase A according to published procedures (Lin et al., 1970; Lin, 1970; Puett, 1972). Just prior to its use, carboxypeptidase A was assayed with hippuryl-L-phenylalanine (Folk & Schirmer, 1963). Stock solutions of RNase 1–118 and of RNase 1–120 were characterized and standardized by amino acid analysis of acid hydrolysates (6 N HCl, 110 °C, 22 h, in vacuo) of aliquot samples; specific activity against cytidine cyclic 2′,3′-phosphate (Murdock et al., 1966) was determined directly in the case of RNase 1–120 and after the addition of a suitable amount of RNase 111–124 in the case of RNase 1–118.

*Synthesis of RNase 111–124.* RNase 111–124, first synthesized by Lin et al. (1970), was prepared by use of solid-phase peptide synthetic methods (Merrifield, 1963; Doscher, 1977; Barany & Merrifield, 1979). Commercial chloromethylated poly(styrene-*co*-1% divinylbenzene) resin (Lab Systems, Inc., lot PMR-16G, 0.74 mmol of Cl/g) was converted to the corresponding hydroxymethylated derivative according to published methods (Gisin & Merrifield, 1972; Wang, 1975). Elemental analysis (Galbraith Laboratories, Inc.) indicated nil Cl in the product. Reaction of a 5.0-g sample of this resin with 1.83 molar excess of Boc-L-valine (Wang, 1975) provided a product containing 0.50 mmol of valine/g. After acetylation of remaining hydroxymethyl groups with pyridine–acetic anhydride (1:1 v/v) (Markley & Dorman, 1970), a standard cycle of deprotection, neutralization, and DCC-mediated coupling (except in the case of Asn-113 where unmediated reaction of the *p*-nitrophenyl ester occurred) (Barany & Merrifield, 1979) was followed for the elaboration of the protected, 14-residue chain (Sasaki, 1981). Removal of the $N^{im}$-DNP group from histidine-119 by thiolysis (Shaltiel & Fridkin, 1970) was followed by acidolysis of the remaining protecting groups, as well as cleavage from the resin with HBr in trifluoroacetic acid (Barany & Merrifield, 1979). The crude peptide was purified to apparent homogeneity by gel filtration on a 2 × 200-cm column of Sephadex G-50, 40–120 μm, followed by ion-exchange chromatography on columns of SP-Sephadex G-25, 40–120 μm (Sasaki, 1981). The yield of final product was 26%. Stock solutions were characterized and standardized by amino acid analysis of acid hydrolysates (6 N HCl, 110 °C, 22 h, in vacuo) of aliquot samples. All stock solutions were stored at −20 °C. Amino acid analyses were performed with a Beckman 120C amino acid analyzer.

*Deuteration of RNase 111–124.* The C(2) position of histidine-119 in the synthetic 111–124 peptide was deuterated by maintaining a 4 mM solution in $D_2O$, pH 8.0, at 40 °C

Table I: Least-Squares Analysis of Histidine pH Titrations

| histidine[a] | sample | $\delta_A$[c] | $\delta_{AH} - \delta_A$ | p$K_a$ | $n$ |
|---|---|---|---|---|---|
| 12 | RNase[b] | 7.35 (0.01) | 1.37 (0.02) | 5.79 (0.07) | 0.67 (0.02) |
| 12 | RNase | 7.66 (0.01) | 1.35 (0.03) | 5.96 (0.02) | 0.70 (0.03) |
| 12 | RNase 1-118·111-124 | 7.67 (0.01) | 1.35 (0.02) | 5.94 (0.02) | 0.69 (0.02) |
| 105 | RNase[b] | 7.39 (0.01) | 1.07 (0.02) | 6.72 (0.02) | 0.94 (0.03) |
| 105 | RNase | 7.63 (0.04) | 1.14 (0.05) | 6.88 (0.05) | 0.82 (0.06) |
| 105 | RNase 1-118·111-124 | 7.69 (0.02) | 1.07 (0.02) | 6.78 (0.02) | 0.89 (0.03) |
| 119 | RNase[b] | 7.45 (0.01) | 1.07 (0.02) | 6.19 (0.04) | 0.79 (0.03) |
| 119 | RNase | 7.75 (0.01) | 1.07 (0.02) | 6.29 (0.01) | 0.79 (0.02) |
| 119 | RNase 1-118·111-124 | 7.76 (0.01) | 1.07 (0.02) | 6.26 (0.02) | 0.77 (0.03) |

[a] Based on the assignment of Markley (1975b). [b] The parameters are taken from Markley (1975b). [c] Markley's values for $\delta_A$ were determined relative to an external standard of 5% $(CH_3)_4Si$ in $CCl_4$ whereas our values are relative to an internal standard of 0.5 mM DSS. A weighted average of the differences between the two sets of values for native ribonuclease indicates that $(CH_3)_4Si$ resonates 0.29 (0.01) ppm downfield from DSS under these experimental conditions.

for 4 days (Meadows et al., 1968).

*NMR Experiments.* The NMR samples of RNase 1–118, RNase 111–124, and RNase 1–118·111–124 were prepared from stock solutions whose concentrations were known from amino acid analyses. Aliquots were lyophilized, reconstituted with $D_2O$, adjusted to pH 3.0 with 2 M DCl and NaOD, and then heated at 60 °C for 1 h to exchange the amide protons (Markley, 1975b). They were then made 0.3 M in NaCl and 0.5 mM in DSS and adjusted to the desired pH. The samples of native RNase A were prepared similarly from Worthington RAF-grade material (phosphate free), but their concentrations were determined spectrophotometrically by assuming an absorption of 0.73 at 280 nm for a 1 mg/mL solution of RNase A (Worthington Enzymes and Related Biochemicals, 1979).

The pH values were determined at room temperature with an Ingold electrode connected to an Altex digital pH meter. Readings were taken before and after the NMR measurements. Whenever possible, the samples were stirred during the pH measurements, but small samples (300 μL) were measured directly in the 5-mm NMR tubes without stirring. The two methods gave the same pH values within experimental error.

The proton NMR spectra were acquired on a Nicolet NT-300 Fourier spectrometer at 30 °C. Most of the spectra extended ±2000 Hz from the carrier frequency, which was set on the HDO resonance, and contained 8192 data points in their free induction decays. Several spectra taken at the beginning of this study used 4096 points or extended ±3012 Hz from the carrier. "Partially relaxed" spectra used a 5.0-μs acquisition pulse with a 2-s delay between pulses; "fully relaxed" spectra used a 5.6-μs acquisition pulse (90°) with 6 s between pulses. For all spectra, the HDO resonance was reduced by homonuclear decoupling, the chemical shifts are reported relative to DSS, and a line-broadening of 1 Hz was applied.

Approximate relative peak areas (Table II) were determined on the NT-300 video display by manually adjusting the parameters of Lorentzian curves to fit the experimental resonance. Nicolet software was used.

## Results

Spectrum 1A (Figure 1) shows the C(2)-$^1$H resonances of the four histidines of native ribonuclease A in 0.3 M NaCl, pH 4.1, at 30 °C. In order of decreasing chemical shift, they have been assigned to histidine-12, -119, -105, and -48 (Meadows et al., 1968; Bradbury & Chapman, 1972; Markley, 1975b). These conditions are the same as those used by Markley (1975b), and in Table I we have compared the parameters that we calculated from least-squares analyses of the pH titration profiles of the histidine resonances (Figure 4) with

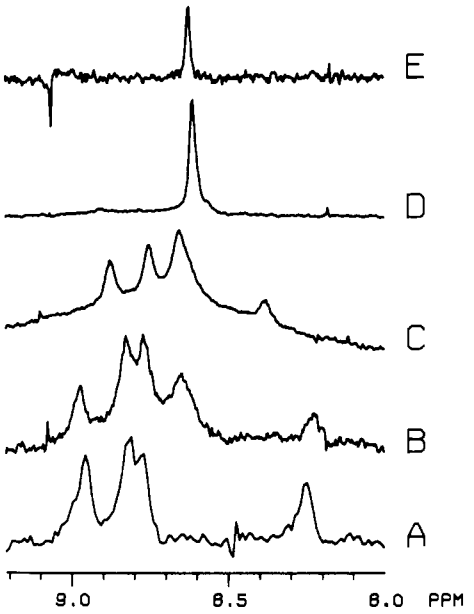


FIGURE 1: Partially relaxed $^1$H spectra at 300 MHz of (A) 2.7 mM RNase A, 512 acquisitions, (B) 2.7 mM RNase 1–118·111–124, 512 acquisitions, (C) 2.8 mM RNase 1–118, 2048 acquisitions, (D) 2.8 mM RNase 111–124, 2048 acquisitions, and (E) 2.8 mM glycylhistidylglycine, 2048 acquisitions; RNase A at pH 4.1 and all other samples at pH 4.0.

those reported by him. The observed chemical shifts, $\delta_{obsd}$, were fit to an equation that incorporated a Hill coefficient, $n$ (Markley, 1975a):

$$\delta_{obsd} = \delta_A + (\delta_{AH} - \delta_A)\frac{[H]^n}{K_a^n + [H]^n} \quad (1)$$

where $\delta_A$ is the chemical shift of the resonance from unprotonated histidine, $\delta_{AH}$ is the chemical shift from protonated histidine, and $K_a$ is the acid dissociation constant. Similarly, spectrum 1B displays the C(2)-$^1$H resonances of the same four histidines in RNase 1–118·111–124, and Table I lists the least-squares parameters for their titration profiles.

The fifth resonance at 8.64 ppm in spectrum 1B was unexpected and prompted the acquisition of the last three spectra in Figure 1, namely, those of RNase 1–118, RNase 111–124 and glycylhistidylglycine, respectively. The spectra in Figure 2 of RNase A and RNase 1–118·111–124 at pH 3.0 further illustrate the nature of the resonance at 8.64 ppm.

The relative areas of the C(2)-$^1$H histidine resonances of RNase 1–118 in a fully relaxed spectrum (not shown, but similar to spectrum 1C) and of RNase 1–118·111–124 (spectrum 3A) are listed in Table II. They were calculated by summing the areas of the resonances, dividing by 3 (his-

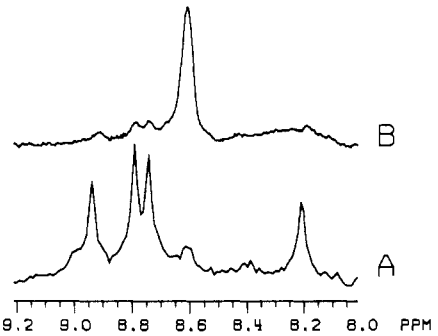


FIGURE 2: Partially relaxed $^1$H spectra at 300 MHz of (A) 2.7 mM RNase A and (B) 2.7 mM RNase 1–118·111–124. Both spectra include 512 acquisitions and are at pH 3.0. The chemical shift scale uses the same offset as later spectra that were taken after the spectra had been made 0.4 mM in DSS and 0.3 M NaCl and is therefore only approximate in these spectra.

Table II: Relative Areas, $A$ (in Protons), of Histidine C(2)-H Resonances of RNase 1–118 and RNase 1–118·111–124

| RNase 1–118 | | | RNase 1–118·111–124 | | |
|---|---|---|---|---|---|
| chemical shift (ppm) | histidine[a] | $A$ | chemical shift (ppm) | histidine | $A$ |
| | | | 8.96 | 12 | 0.53 |
| 8.87 | 12 | 0.63 | 8.81 | 119 | 1.35 |
| 8.75 | 105 | 0.51 | 8.76 | 105 | 0.65[b] |
| 8.65 | denatured | 1.29 | 8.64 | denatured | 0.94 |
| 8.37 | 48 | 0.13 | | | |
| | | | 8.22 | 48 | 0.31 |

[a] The assignments are by analogy with the relative chemical shifts in RNase A and are not definitive. [b] It overlaps with a histidine resonance of dissociated RNase 1–118.

tidines) for RNase 1–118 or 4 for the complex, and then taking the ratios with the areas of the individual resonances. For reasons that are presented under Discussion, the correct signal for histidine-48 in RNase 1–118 was assumed to be equal to the average of the areas of histidine-12 and -105; in RNase 1–118·111–124, it was assumed to be equal to the area of histidine-12.

Because the assignments of the resonances of histidine-12 and -119 had been reversed from the original assignments (Markley, 1975b), we have used our semisynthetic system to check the assignment of histidine-119 in RNase 1–118·111–124 by using RNase 111–124 in which the C(2) position of histidine-119 had been deuterated. The spectra are shown in Figure 3. As this experiment required accurate relative areas for the resonances, we measured approximate spin–lattice relaxation times ($T_1$) for samples of RNase 1–118, RNase 1–118·111–124, and RNase 111–124. The largest $T_1$ value observed, 0.9 ± 0.2 s, belonged to histidine-48 in RNase 1–118. Consequently, we used a delay of 6 s between each acquisition pulse for the spectra in Figure 3. The longest $T_1$ reported by Benz et al. (1972) for RNase A at 100 MHz was 0.98 s for histidine-105. Westmoreland & Matthews (1973) determined an upper limit of 1.4 s for the $T_1$ values of the native and denatured histidine resonances of RNase A.

## Discussion

Spectra 1A and 1B indicate that in RNase 1–118·111–124 the four histidine residues have essentially the same environments as they do in RNase A. This observation correlates with the facts that RNase contains two active site histidine residues (histidine-12 and histidine-119) (Wyckoff & Richards, 1971) and the semisynthetic complex is 98% active. Table I shows

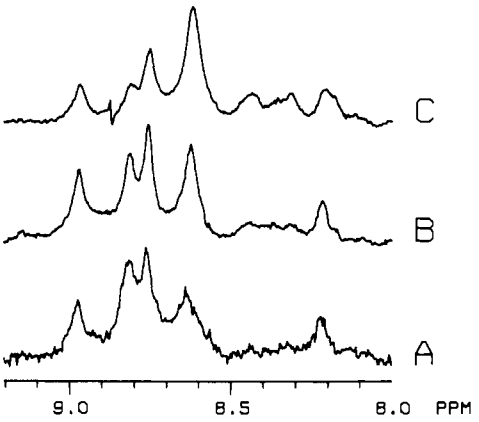


FIGURE 3: Fully relaxed $^1$H spectra at 300 MHz showing titration of RNase 1–118·111–124 with deuterated RNase 111–124 at pH 4.0: (A) 2.7 mM RNase 1–118·111–124 and no deuterated RNase 111–124; (B) 1.9 mM RNase 1–118·111–124 + 1.9 mM deuterated RNase 111–124; and (C) 1.6 mM RNase 1–118·111–124 + 4.8 mM deuterated RNase 111–124. The spectra include 512, 2048, and 4096 acquisitions, respectively.

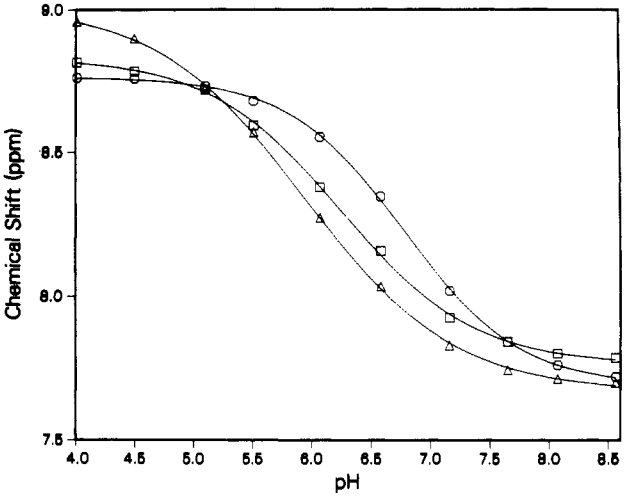


FIGURE 4: Chemical shifts, as a function of pH, of resonances of histidine-12 (△), histidine-105 (○), and histidine-119 (□) of RNase 1–118·111–124 at 30 °C in 0.3 M NaCl. The curves were calculated from the least-squares parameters in Table I.

that the similarity extends over the complete titration range: the acid dissociation constants, the chemical shifts of the protonated and unprotonated forms, and the Hill coefficients for histidine-12, -105, and -119 correspond between RNase A and the semisynthetic complex. However, the p$K_a$ values in Table I do indicate that there is a systematic difference between our pH measurements and those of Markley (1975b) of about 0.1 pH unit. Histidine-48 does not appear in Table I because it has a discontinuous titration curve in both RNase A and RNase 1–118·111–124. Markley (1975c) has presented evidence that histidine-48 titrates only as the result of a slow conformational transition; apparently, this transition also occurs in the semisynthetic enzyme.

The resonance at 8.64 ppm in spectrum 1B of RNase 1–118·111–124 was unexpected. Examination of the spectra of RNase 1–118 (1C), of RNase 111–124 (1D), and of glycylhistidylglycine (1E) suggests that the resonance at 8.64 ppm is that of "unstructured" histidine that is in slow exchange with the separate histidine C(2)-$^1$H resonances of the nondenatured species. It is presumably observed in spectra 1B and 1C because RNase 1–118 is less stable than RNase under the conditions used for spectra acquisition; Lin (1970) reported transition temperatures at pH 7.5 for RNase 1–118 and

RNase of 32.5 and 61.0 °C, respectively. This assignment is reinforced by spectrum 2B, which shows a marked increase in the amount of free or unstructured histidine at pH 3.0 relative to the amount of structured histidine. Native RNase A will denature fully at pH 1.0 (Wyckoff & Richards, 1971); spectrum 2A indicates that a small amount of unstructured histidine exists in RNase A, pH 3.0. Westmoreland & Matthews (1973) observed this same resonance in their study of the thermal denaturation of RNase at pH 1.3 and ascribed it to histidine in a denatured structure. Although RNase 1–118 (and, consequently, RNase 1–118·111–124) is less stable than RNase A at 30 °C, no unstructured histidine is evident in spectra taken above pH 5.5 (not shown); this observation also argues against the resonance at 8.64 in spectra 1B and 3A being a result of excess RNase 111–124.

Alternatively, the resonance at 8.64 ppm could be a superposition of several histidine resonances from a stable, partially unfolded structure of RNase 1–118 and perhaps of RNase 1–118·111–124 as well. Biringer & Fink (1982) used 35–50% methanol to stabilize such intermediates of RNase A (and thereby greatly decreased the cooperativity of the transition between the native and unfolded states). They observed separate histidine C(2)-$^1$H resonances from a partially folded intermediate in the kinetic refolding of RNase A at −16 °C, 35% methanol, pH 2.8, and also from a less compact but still partially folded intermediate in the equilibrium unfolding of RNase A at 33 °C, 50% methanol, pH 3.0. The four separate histidine resonances from the latter intermediate lay between about 8.57 and 8.66 ppm (their spectrum 2D); no resonance was observed from fully unfolded RNase A at the conditions of the experiment.

We consider it unlikely that any partially folded intermediates contribute significantly to the resonance at 8.64 ppm in our experiments. In the absence of methanol, the transition from the native to the unfolded state is highly cooperative (Blum et al., 1978; Biringer & Fink, 1982), and no significant population of intermediates should exist when RNase unfolds under equilibrium conditions; a comparable degree of cooperativity was observed for RNase 1–118 (Lin, 1970). The NMR spectra of Matthews & Westmoreland (1973) showed only a single transition curve for the unfolding of the histidine C(2) $^1$H of RNase A at pH 4.0 in aqueous solution. Our spectra of RNase 1–118 and RNase 1–118·111–124 show no resonances separate from but close to that at 8.64 ppm, which could be construed to belong to the slowly exchanging intermediate studied by Biringer & Fink. If the intermediate is in fast exchange, there is not enough of it to shift the resonances of either the folded or unfolded species significantly away from their expected positions.

It is possible, of course, that other intermediates exist in the absence of methanol that are less compact than those investigated by Biringer and Fink and that, therefore, have histidine resonances that are not resolved from those of fully unfolded RNase. However, the kinetic intermediates observed in methanol solutions form rapidly from the unfolded species (Biringer & Fink, 1982), and we expect that any less structured intermediates in our experiments would likewise be in rapid equilibrium with the unfolded species. Consequently, such unresolved intermediates are equivalent to, and have been subsumed with, the completely unfolded species in our analysis of the equilibrium unfolding of RNase 1–118 and RNase 1–118·111–124.

The effect of additions of RNase 111–124 in which the C(2) position of histidine-119 had been deuterated is demonstrated in the spectra of Figure 3. The deuterated peptide exchanges with the original protonated material, thus decreasing the resonance of histidine-119 (8.81 ppm) on the enzyme and increasing the resonance of unstructured histidine. This experiment agrees with the corrected assignment of histidine-119 (Markley, 1975b) and our interpretation of the resonance at 8.64 ppm in spectra 1B and 3A. The ratio between total peptide and RNase 1–118 in spectra 3A–3C is 1, 2, and 4, respectively. At the higher ratios, additional resonances of unknown origin appear between unstructured histidine at 8.64 ppm and histidine-48 at 8.22 ppm. As they are not seen in the spectrum of RNase 111–124 alone (1D), they must derive from some interaction between the excess peptide and the complex. They are unlikely to be histidine resonances as the protonated RNase 111–124 goes to the unstructured histidine position when it is exchanged off the complex.

With some simplifying assumptions, the spectra in Figures 1 and 3 can be used to estimate a binding constant for the association of RNase 1–118 and RNase 111–124 at pH 4.0. Spectrum 3A, of 2.7 mM RNase 1–118·111–124, contains histidine C(2)-$^1$H resonances from a mixture of the complex, some dissociated RNase 1–118, and an equal amount of dissociated RNAse 111–124. Spectrum 1C indicates that the dissociated RNase 1–118 exists in an equilibrium between structured and denatured forms, while spectrum 1D suggests that the dissociated RNase 111–124, or at least histidine-119 on it, exists only in a denatured form. Consequently, we assume the following equilibria

$$\text{RNase* 1–118} \overset{K_1}{\rightleftharpoons} \text{RNase 1–118} + \text{RNase* 111–124} \overset{K_2}{\rightleftharpoons} \text{RNase 1–118·111–124} \quad (2)$$

where the asterisk marks a denatured state. Earlier studies on RNase (Roberts & Benz, 1973; Westmoreland & Matthews, 1973) have shown that the local structures around the histidine denature at different rates. Consequently, other intermediate states may exist but are ignored here to facilitate the analysis. The average of the mole fractions of histidine-12 and -105 in spectrum 1C can then be taken to be that of RNase 1–118; similarly, the mole fraction of histidine-12 in spectrum 3A, whose resonance does not overlap with any resonance of RNase 1–118, RNase* 1–118, or RNase* 111–124, can be equated with that of the complex, RNase 1–118·111–124.

The anomalous behavior of histidine-48 cannot be ignored in making the calculation; its relative area in both the complex and in RNase 1–118 is significantly lower than that of the other histidines (Table II). In spectra of RNase A, the area of the assigned resonance of histidine-48 equals one proton below pH 5.0, but above that pH, histidine-48 passes through one or more intermediates, whose resonances are undetectable, to a high pH conformation with a known chemical shift (Markley, 1975c). Consequently, for RNase A, the areas of the assigned resonances of histidine-48 do not sum to one proton between pH 5 and 7. For RNase 1–118 and RNase 1–118·111–124 at pH 4.0, the relative area of the resonance assigned to histidine-48 is also significantly lower when compared with those of the other histidines. Two interpretations are possible: (1) histidine-48 denatures as easily as the other histidines, and the missing signal belongs to unobserved intermediates similar to those that occur in RNase at higher pH values; (2) histidine-48 simply denatures more readily than do the other histidines, and the missing signal is actually at 8.64 ppm. We consider the first interpretation to be more likely. Histidine-48 is the most buried histidine, as reflected by its resistance to deuteration (Markley, 1975b). In addition, two studies indicate it is among the least likely of the four

histidines to denature (Roberts & Benz, 1973; Westmoreland & Matthews, 1973). Finally, if the 37 aromatic resonances of RNase 1–118, which has more signal missing for histidine-48 than does RNase 1–118·111–124, are integrated and normalized to give the area of one proton, the total observed histidine signal in spectrum 3C is less than the three protons required by the second interpretation.

The relative areas, in protons, of the resonances in spectra 1C and 3A are listed in Table II. The stability constant, $K_1$, is calculated from the data of RNase 1–118 alone (i.e., spectrum 1C), by assuming the mole fraction of RNase 1–118, $f_{1\text{-}118}$, is equal to the average relative areas of histidine-12 and -105:

$$f_{1\text{-}118} = 0.5(A_{H12} + A_{H105}) = 0.57 \quad (3)$$

$$K_1 = \frac{[\text{RNase 1–118}]}{[\text{RNase* 1–118}]} = \frac{f_{1\text{-}118}}{f^*_{1\text{-}118}} = \frac{f_{1\text{-}118}}{1 - f_{1\text{-}118}} = 1.33 \quad (4)$$

$K_1$ may also be calculated from the area of the resonance of denatured histidine. As explained above, we assume that all three histidines are denatured to the same degree:

$$3f^*_{1\text{-}118} = A_{\text{unstr}} \quad (5)$$

$$f^*_{1\text{-}118} = 1.29/3 = 0.43$$

$$K_1 = \frac{1 - f^*_{1\text{-}118}}{f^*_{1\text{-}118}} = 1.33 \quad (6)$$

$K_2$ is calculated from $K_1$ and the relative area of histidine-12 in spectrum 3A of the complex:

$$f_{1\text{-}118\cdot111\text{-}124} = A_{H12} = 0.53 \quad (7)$$

$$f_{1\text{-}118} + f^*_{1\text{-}118} = 1 - f_{1\text{-}118\cdot111\text{-}124} = 0.47 \quad (8)$$

But, from eq 4

$$f_{1\text{-}118} = K_1 f^*_{1\text{-}118}$$

so

$$f_{1\text{-}118} = 0.27 \qquad f^*_{1\text{-}118} = 0.20 \quad (9)$$

Also, because the two fragments are equimolar

$$f_{111\text{-}124} = f_{1\text{-}118} + f^*_{1\text{-}118} \quad (10)$$

so

$$K_2 = \frac{[\text{RNase 1–118·111–124}]}{[\text{RNase 1–118}][\text{RNase 111–124}]} = \frac{f_{1\text{-}118\cdot111\text{-}124}}{f_{1\text{-}118} f_{111\text{-}124}(2.7 \times 10^{-3}\ \text{M})} = 1.5 \times 10^3\ \text{M}^{-1} \quad (11)$$

$K_2$ can also be calculated from the area of the resonance of unstructured histidine at 8.64 ppm. In terms of protons

$$3f^*_{1\text{-}118} + f_{111\text{-}124} = 0.94 \quad (12)$$

Substituting eq 4 and 10, and the value of $K_1$, we obtain

$$f_{111\text{-}124} = 0.41 \qquad f_{1\text{-}118} = 0.23 \qquad f^*_{1\text{-}118} = 0.18$$

$$f_{1\text{-}118\cdot111\text{-}124} = 0.59$$

These values give a $K_2$ of $2.3 \times 10^3\ \text{M}^{-1}$ from eq 11, which agrees reasonably well with the preceding value of $1.5 \times 10^3\ \text{M}^{-1}$. Their average value is $1.9 \times 10^3\ \text{M}^{-1}$. If the second interpretation of the behavior of histidine-48 is chosen, then $K_1$ is 2.0, and the average $K_2$ is $1.4 \times 10^3\ \text{M}^{-1}$.

These binding constants are measured at pH 4.0 in the absence of substrate; Gutte et al. (1972) obtained a value of $5 \times 10^6\ \text{M}^{-1}$ for $K_2$ in the presence of substrate at pH 6.0. It is reasonable that substrate should stabilize the semisynthetic complex and increase the association constant. Furthermore, our data indicate that increasing pH also stabilizes RNase 1–118·111–124, in that no unstructured histidine resonance is observed above pH 5.5.

The assumptions that underlie this analysis of the equilibria allow us to calculate equilibrium constants that we believe are approximately correct, but they do not allow us to explain some of the details of the spectrum of RNase 1–118·111–124 (spectrum 3A). In particular, the mole fractions calculated above predict a significant peak (0.23 proton) at 8.87 ppm from the dissociated RNase 1–118 in the sample. This resonance is not seen. A possible explanation is that the magnitude of the histidine-12 resonance underestimates the amount of RNase 1–118·111–124 and therefore overestimates the amount of RNase 1–118; alternatively, the resonance at 8.87 ppm predicted for RNase 1–118 may be shifted or broadened by, respectively, a fast or intermediate exchange process in the presence of RNase 111–124.

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**Registry No.** RNase 1–118, 86176-83-2; RNase 111–124, 55247-96-6; His, 71-00-1.

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# Auxin-Induced Changes in the Level of Translatable Ribosomal Protein Messenger Ribonucleic Acids in Soybean Hypocotyl†

J. Stephen Gantt* and Joe L. Key

ABSTRACT: Two-dimensional polyacrylamide gel electrophoretic maps of ribosomal proteins isolated from soybean seedlings were constructed prior to the investigation of mRNA levels. Polysomes and ribosomal subunits were isolated from the apical hooks of 3-day-old soybean seedlings. A total of 40 and 51 basic proteins, associated with the 40S and 60S subunits, respectively, was resolved on the four different two-dimensional gel systems used in this study. The effect of the synthetic auxin (2,4-dichlorophenoxy)acetic acid (2,4-D) on ribosomal protein mRNA levels was investigated by in vitro translation of RNA isolated from 2,4-D-treated and nontreated hypocotyls. The in vitro synthesized proteins were extracted and separated by two-dimensional polyacrylamide gel electrophoresis. Stained protein spots that corresponded to ribosomal proteins were excised from the gel, and the in vitro incorporation of [$^{35}$S]methionine was determined. Results indicate that auxin induces an 8-fold increase in the relative amount of translatable ribosomal protein mRNAs.

Auxins are a class of naturally occurring plant growth regulators that are required for both cell division and expansion (Thimann, 1969; Meins, 1977). There is a great deal of information that supports the hypothesis that the physiological effects of auxin result from altered gene expression. Some 10–12 h after the application of auxin to young soybean hypocotyls, cell division commences in this normally quiescent tissue (Key et al., 1966). However, prior to cell proliferation, there is a massive accumulation of protein and RNA. Early studies used specific inhibitors of protein and RNA metabolism to link the influence of auxin to changes in gene expression. Inhibition of RNA synthesis by actinomycin or of protein synthesis by cycloheximide results in an inhibition of auxin-induced cell elongation (Key & Ingle, 1964, 1967; Key et al., 1967). Hybridization analyses have shown that the levels of few of the approximately 40 000 poly(A)-containing RNA species are significantly altered by auxin (Baulcombe et al., 1980, 1981). In vitro translation of poly(A)-containing RNA followed by two-dimensional gel electrophoresis reveals that auxin significantly alters only 5–10% of the abundant mRNA species (Baulcombe et al., 1980). Zurfluh & Guilfoyle (1982a) have shown that auxin induces changes in the levels of about ten translatable mRNAs in the elongating section of soybean hypocotyl tissue. It also alters the levels of approximately 20 translatable mRNAs in both excised basal sections and intact soybean hypocotyl (Zurfluh & Guilfoyle, 1982b). Theologis & Ray (1982) have made similar observations using poly(A)-containing RNA isolated from excised pea epicotyl tissue. Using cloned cDNA, Walker & Key (1982) have directly demonstrated that auxin induces changes in the level of two mRNAs found at high levels in the elongating section of the soybean hypocotyl. One mRNA increases 3–5-fold while the other increases 5–8-fold. These mRNAs are quickly induced, which suggests that auxin directly affects their expression.

Auxin treatment of soybean hypocotyl results in large increases in rRNA production (Key et al., 1966). Olszewski & Guilfoyle (1980) have shown that by 24 h auxin-treated hypocotyls have 9-fold more template-engaged RNA polymerase I activity per amount of DNA than does control hypocotyls. This change in the rate of rRNA synthesis is the result of an increase in the rate of both initiation and elongation by RNA polymerase I. In artichoke explants, enhanced RNA polymerase I activity and an increased conservation of rRNA sequences during pre-rRNA processing account for the observed increase in rRNA synthesis (Melanson & Ingle, 1978).

The increase in rRNA production and the concomitant accumulation of ribosomes following auxin treatment suggests that ribosomal proteins may also be produced at an elevated rate. Two mechanisms have been described that account for increased amount of ribosomal protein accumulation in higher eukaryotes. Nabeshima & Ogata (1980) demonstrated that the in vivo rate of ribosomal protein synthesis in regenerating rat liver was selectively increased by a factor of 3 compared to that in normal rat liver. Furthermore, in a cell-free translation system, the elevated rates of synthesis were shown

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