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SYNTHESIS OF RIBONUCLEASE LABELLED WITH ^{13}C ON METHIONINE-29

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SUMMARY

The C^{ε} methyl group of methionine-29 of RNAase was enriched with ^{13}C. The synthesis involved the reaction of RNAase with $^{13}CH_3I$ at pH 4. S-Methyl-methionine-29 RNAase was recovered in 80% yield. This sulfonium derivative was subsequently demethylated with 0.1 M mercaptoethanol at pH 8.5, 25°C for 4 days. These conditions allowed the demethylation reaction to successfully compete with the reaction of the thiol with the four disulfide bridges in RNAase. After dialysis, concentration and chromatography, native RNAase with approx. 50% of its Met^{29} methyl groups enriched in ^{13}C was recovered as was unreversed S-Methylmethionine-29 RNAase. Both proteins showed full enzymatic activity toward cytidine 2':3'-cyclic monophosphate. ^{13}C-methyl signals from enriched RNAase and the sulfonium derivative were observed at 13.8 and 26.7 ppm from TMS respectively. Preliminary denaturation studies with the methylated protein suggest that ^{13}C enrichment of methionine methyl groups in RNAase will be a useful technique for following the unfolding transition at these sites of the protein.

INTRODUCTION

Nuclear magnetic resonance spectroscopy is a useful technique for obtaining information about the structure and dynamics of biological macromolecules in solution (1).

We and others have studied the behavior of the four assigned histidine $C^{\varepsilon 1}$-H resonances in the ^1H NMR spectrum of RNAase during denaturation induced by a variety of methods (2-4). In order to extend these studies it will be necessary to observe other single amino acid residues located in other regions of the RNAase molecule. Unfortunately, none of the other amino acids in RNAase provide ^1H NMR resonances which are sufficiently well resolved and/or assigned such that they can be readily used for this purpose. Introduction of NMR probes via chemical modification at selected amino acid sites is one possible solution.

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Our initial objective was to selectively label one of the four methionines in RNAase with ^{13}C, specifically C^{ε} of Met^{29}. This goal was based on the knowledge that treatment of RNAase with methyl iodide (CH_3I), under appropriate conditions, leads to the selective formation of the methyl sulfonium salt of Met^{29} (5). Reaction with $^{13}CH_3I$ would therefore generate a selectively labelled derivative. However, this derivative is not RNAase since it contains one additional methyl group and, more importantly, an extra positive charge on a residue which is normally uncharged. The observation by Naider and Bohak that methionine residues can be regenerated from their sulfonium salts by treatment with nucleophiles, such as mercaptoethanol (6), suggested that the sulfonium derivative [MethylMet^{29}-$^{13}C^{\varepsilon}$] RNAase could be converted back into native RNAase by mercaptoethanol. The regenerated RNAase would now be partially enriched in ^{13}C at C^{ε} of methionine-29. This synthesis strategy has been successfully used by Jones et al. to enrich C^{ε} of the two methionine residues of sperm whale myoglobin (7) and by Schejter et al. on cytochrome C (8).

Unlike myoglobin and cytochrome C, however, RNAase contains four disulfide bridges which could be reduced by the thiol, leading to a disruption of the enzyme's tertiary structure. Thus, application of previous techniques to RNAase is not straightforward. Fortunately, RNAase is relatively stable to thiols in the absence of denaturants (9-10). This suggested that it might be possible to find conditions which would selectively demethylate [MethylMet^{29}-$^{13}C^{\varepsilon}$] RNAase with minimal disulfide bond reduction.

MATERIALS AND METHODS

Bovine pancreatic ribonuclease (Worthington grade RAF, phosphate free) was used without further purification. Other reagents and their sources were as follows: Cytidine 2':3'-cyclic monophosphoric acid and 5,5'-dithiobis (2-nitrobenzoic acid) from Sigma Chemical Co.; ^{13}C enriched methyl iodide (92%) and Biorex 70 from Bio-Rad Laboratories; 2-mercaptoethanol and sodium deuteroxide from Aldrich Chemical Co; Ultra Pure guanidine hydrochloride from Schwarz-Mann; deuterium oxide, acetic acid-d_4 and deuterium chloride from Stohler Isotope Chemicals.

RNAase Assays: The hydrolysis of cyclic-CMP was measured by the standard assay described by Crook et al. (11).

Methylation of Methionine-29 in RNAase with $^{13}CH_3I$: One hundred milliliters of RNAase (3 mg/ml) in 0.1 M KNO_3 was placed in a 100 ml volumetric flask and

the pH was adjusted to pH 4-5 with 6 N HCl. Two grams of $^{13}CH_3I$ were added and the flask was capped tightly. The two phase mixture was stirred for 24 hours at 25°C in the dark. At the end of this period, the solution was transferred to a lyophilization flask and the unreacted methyl iodide was removed under gentle vacuum. The pH of the solution was raised to pH 6.0 with 1.0 N NaOH. The sample was concentrated from 100 ml to 30 ml in a Millipore molecular filtration vessel. The concentrated mixture was fractioned in three batches (10 ml - 100 mg protein) on a column of Biorex-70 (200-400 mesh) (2.5 x 97 cm) at 4°C. The products were eluted with 0.2 M phosphate, pH 6.3 at a flow rate of 60 ml/hr.

Demethylation of S-Methylmethionine-29 RNAase: The fractions containing the methylated RNAase derivative were pooled. The pH, protein and mercaptoethanol concentrations were adjusted to pH 8.5, 1.8 x 10^{-5} M and 0.1 M, respectively. The protein was assayed for enzyme activity and thiol concentration daily. After 4 days at 25°C, the pH of the solution was lowered to pH 6.0 with 6 N HCl. The solution was then dialyzed against deionized water at 4°C in a Biofiber 50 dialysis beaker (Biorad). The dialysis of each batch was stopped when the thiol concentration had been lowered 100 fold. The dialyzed protein was then transferred to a rotary evaporator and concentrated to approx. 150 ml. The protein solvent was exchanged for 0.1 M KNO_3 in the Biofiber 50 beaker at 4°C and the solution was subsequently concentrated in the Millipore cell and fractionated as described above in the methylation procedure. The fractions containing the regenerated RNAase and S-Methylmethionine-29 RNAase which survived the demethylation reaction were pooled separately, concentrated in the rotary-evaporator and dialyzed against deionized water at 4°C. The products were then lyophilized and stored frozen pending NMR analysis.

NMR Spectroscopy: To prepare the samples for NMR spectroscopy, the proteins were dissolved in 0.2 M deuteroacetate buffer. The pH was measured with a Radiometer model 20 PHM 64 pH meter with a Beckman 39030 combination electrode. ^{13}C NMR spectra were recorded with a Bruker HX90-E Fourier transform spectrometer operating at 22.63 MHz. The instrument was field-frequency locked to the deuterium in the solvent. The spectra were collected in a Nicolet 1080 computer. All samples were placed in a 0.43 ml microcell (Wilmad) within a 10 mm O.D. NMR tube. Trace amounts of dioxane (approx. 5 mM) were added to each sample to serve as an internal chemical shift reference (67.8 ppm downfield of TMS).

RESULTS AND DISCUSSION

[MethylMet^{29}-$^{13}C^{\varepsilon}$] RNAase was prepared by reacting RNAase with $^{13}CH_3I$ and was separated from unreacted RNAase by cation exchange chromatography. A typical profile for this chromatographic step is shown in Fig. 1A. The profile is the same as that obtained by Link and Stark (5) and therefore we can assign the peaks at fraction number 38, 66 and 84 to KNO_3, unreacted RNAase and [MethylMet^{29}-$^{13}C^{\varepsilon}$] RNAase, respectively. The very small peak at fraction 103 corresponds to methylmethionine-29, 79 RNAase (12). The average yield in the methylation reaction was 75-80%.

The fractions representing the sulfonium derivative were pooled and treated with 0.1 M mercaptoethanol as described in the Methods. The chromatographic

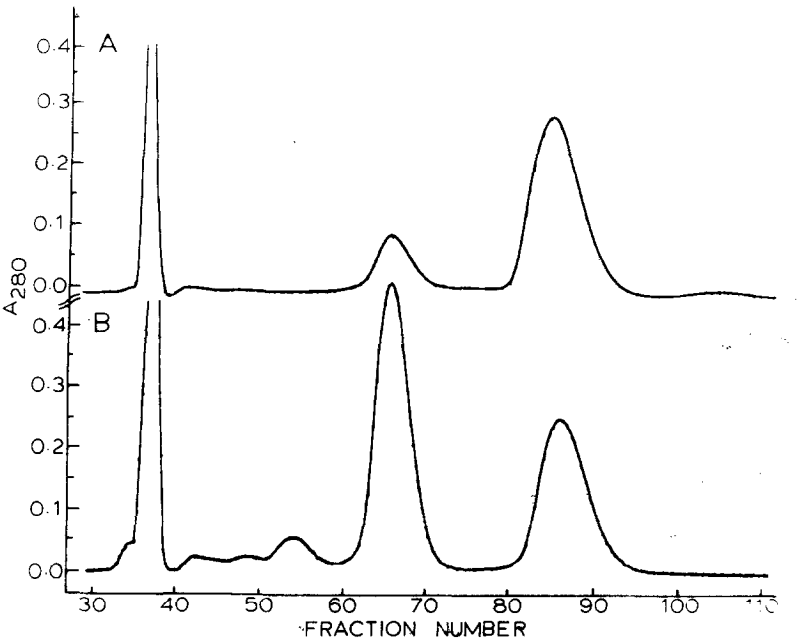

Fig. 1. Cation exchange chromatography of RNAase and derivatives on a column (2.5 x 97 cm) of Biorex-70, 0.2 M phosphate, pH 6.3, 4°C, 60 ml/hr. Fig. 2A shows the separation of unreacted RNAase (Fraction 66) from [MethylMet^{29}-$^{13}C^{\varepsilon}$] RNAase (Fraction 84). Fig. 2B shows the separation of [Met^{29}-$^{13}C^{\varepsilon}$] RNAase (Fraction 66) from [MethylMet^{29}-^{13}C] RNAase. See text for further details.

profile obtained after the demethylation step is shown in Fig. 1B. Fractions 38 and 84 are assigned as described above. Fraction 66, however, now represents [Met^{29}-$^{13}C^{\varepsilon}$] RNAase. Both fractions 66 and 84 show full enzymatic activity toward cyclic CMP. The small peaks at fractions 49 and 54 represent small amounts of side reaction products (13). The profile indicates that the reversal conditions chosen led to considerable demethylation of the sulfonium derivative (50-70% on average). These demethylation conditions are obviously a compromise. Numerous trial conditions not discussed here showed that higher thiol concentrations resulted in a decrease in RNAase activity and longer incubation times in 0.1 M mercaptoethanol led to significant side reactions (13).

Figure 2A shows the spectra obtained from a sample of [MethylMet^{29}-$^{13}C^{\varepsilon}$] RNAase and native RNAase. The resonance at 26.7 ppm from TMS is assigned to

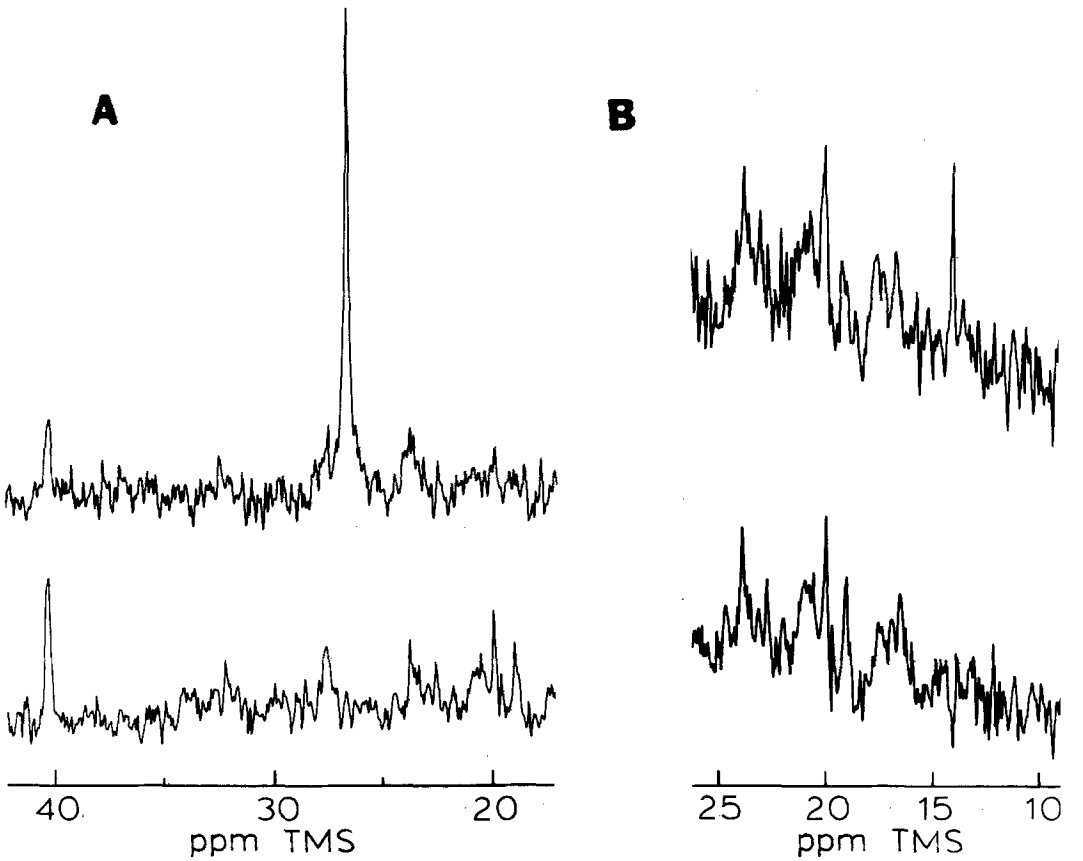

Fig. 2. Proton decoupled Fourier transform ^{13}C NMR spectra of native RNAase and a ^{13}C labelled derivatives. A-top: [MethylMet^{29}-$^{13}C^{\varepsilon}$] RNAase, 5.6 mM in 0.2 M deuteroacetate buffer, pH 6.3, 37°C. Transients = 13,171; repetition rate = 0.82 sec; 8K data points, exponential multiplication of the free induction decay introduced 1.16 Hz broadening. A-bottom: Native RNAase, 4.7 mM in 0.2 M deuteroacetate buffer, pH 6.7, 30°C. Transients = 50,000. B-top: [Met^{29}-$^{13}C^{\varepsilon}$] RNAase 4.7 mM in 0.2 M deuteroacetate buffer, pH 6.7, 30°C. Transients = 50,000. B-bottom: same as A-bottom.

the enriched S+-$^{13}CH_3$ group. The chemical shift of this carbon is pH independent between pH 5-8. This signal is slightly downfield of the equivalent resonance either in a sample of free S-methylmethionine or in denatured [MethylMet^{55,131}-$^{13}C^{\varepsilon}$] sperm whale myoglobin, both of which occur at 26.3 ppm (7). The resonance at 40.4 ppm has been assigned by others (14) to C^{ε} of the ten lysine residues in RNAase.

Subsequent to the demethylation reaction, the resonance at 26.7 ppm disappears and a new resonance at 13.8 ppm appears (Fig. 2B). This signal is

assigned to the enriched methionine-29 C^{ε} carbon of native RNAase. Its chemical shift is 1.4-1.6 ppm upfield of the C^{ε} carbon in free methionine and in the peptide GlyGlyMetGlyGly which occur between 15.2-15.4 ppm from TMS ((7) and F. W. Benz, unpublished observations).

To test the feasibility of using ^{13}C NMR to study the behavior of individual methionine residues in RNAase during an equilibrium denaturation experiment, we treated the sample of [MethylMet^{29}-$^{13}C^{\varepsilon}$] RNAase with guanidine deuterochloride. Our eventual goal is to study this denaturation process with labelled RNAase rather than the sulfonium derivative, however, we chose the latter protein at present because of the considerably larger amounts in hand. Figure 3 illustrates the behavior of the C^{ε} sulfonium carbon at three concentrations of guanidine. Previous ^{1}H NMR studies on the unfolding of RNAase have shown that the major unfolding transition occurred between 2.0-3.3 M guanidine (3). Thus at 1.9 M guanidine, a single resonance for [MethylMet^{29}-$^{13}C^{\varepsilon}$] RNAase in the native state is observed at 26.9 ppm while at 3.1 M a single resonance at 26.3 ppm is seen for the same carbon in the denatured state. In 2.5 M guanidine both states are present and observed to be in slow exchange on the NMR time scale with an exchange rate of less than 92 sec^{-1}. It is interesting to note that the chemical shift of this carbon in the denatured state (3.1 M GuDCl) is identical to that reported by Jones et al. (7) for denatured myoglobin and free S-methylmethionine. In the native state, the RNAase sulfonium signal is even further downfield. In contrast, the signal from [Met^{29}-$^{13}C^{\varepsilon}$] RNAase is upfield of the free amino acid shift. This suggests that the C^{ε} carbon of Met^{29} does not occupy a similar position above Phe^{46} in both the sulfonium derivative and the native enzyme. This is not surprising since, in the sulfonium form, the sulfur carries a positive charge and should move away from Phe^{46} toward the solvent whereas uncharged Met^{29} would do the opposite. Despite the fact that the sulfonium C^{ε} shift suggests that it is more exposed to the solvent in the folded state than is the [Met^{29}-$^{13}C^{\varepsilon}$] carbon in native RNAase, it still is able to monitor the

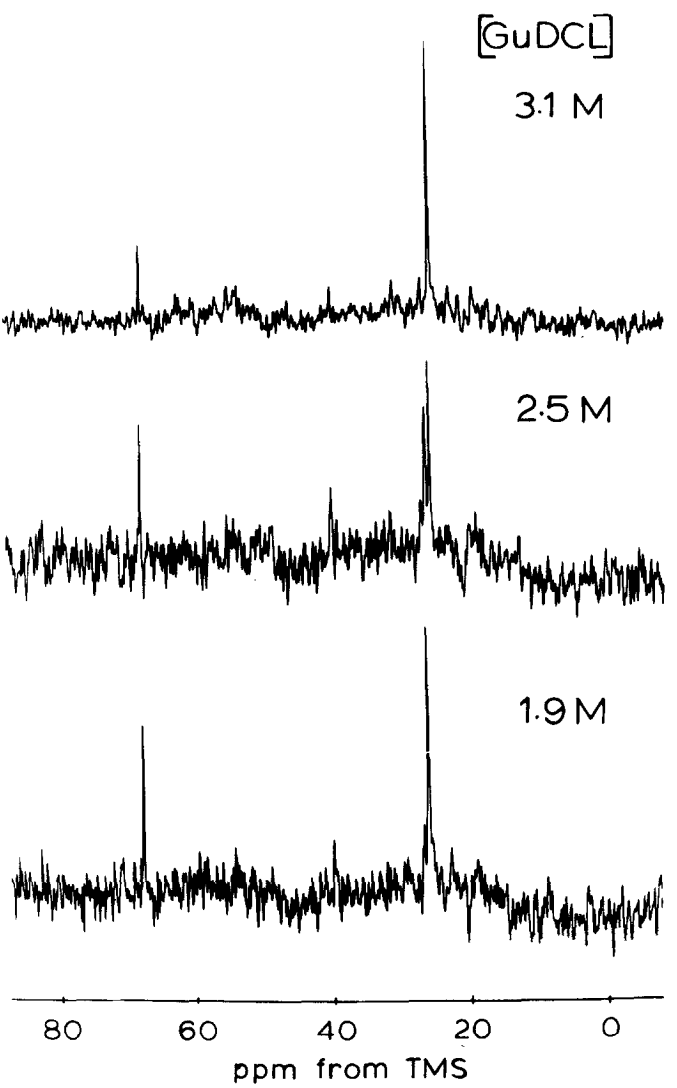

Fig. 3. Proton decoupled Fourier transform ^{13}C NMR spectra of [MethylMet^{29}-$^{13}C^{\varepsilon}$] RNAase, 2 mM in 0.2 M deuteroacetate buffer pH 5.5, 30°C at three concentrations of guanidine deuterochloride (GuDCl). Spectral conditions were as in Fig. 2 except that 24,000 transients were accumulated and the free induction decay was multiplied by an exponential function which introduced 2.33 Hz broadening.

unfolding transition in this area of the polypeptide chain. This strongly suggests that native RNAase labelled at all four methionines would be a useful derivative for following the unfolding behavior and solution dynamics of the methionine regions of RNAase. We are presently in the process of synthesizing this derivative.

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