Preparation and Properties of Three Specific Active Derivatives of Ribonuclease A Obtained by Methylation of Methionine Residues in 8 *M* Urea†

George R. Stark and Thomas P. Link*,‡

ABSTRACT: In 8 *M* urea at low pH, CH_3I reacts specifically with the four methionine residues of ribonuclease A, and all four residues react at the same rate. Upon removal of the denaturant, only unmodified ribonuclease and 3 of the 15 possible derivatives modified on methionine refold to regenerate activity. All the enzymatic activity is recovered after chromatography on IRC-50 and the four active proteins separate from each other and from the 12 inactive derivatives, which are not eluted from the resin under the conditions used. By the use of $^{14}CH_3I$, performic acid oxidation, chymotryptic digestion, and separation of the resulting peptides by ion exchange, the active species were determined to be unmodified ribonuclease, CH_3Met-29-RNase, CH_3Met-79-RNase, and CH_3Met-29, CH_3Met-79-RNase. These proteins have melting temperatures of 63, 58, 43, and 36°, respectively, at pH 6.3–7.0. Methylation at methionine-29 or -79 has no effect on enzymatic activity. Conversely, methylation at methionine-13 or -30 prevents refolding to an active conformation at 25° and elution from IRC-50. These results are consistent with the positions of the four methionine residues in crystals of ribonuclease A and ribonuclease S as determined by X-ray diffraction.

Bovine pancreatic ribonuclease A has four methionine residues among its 124 amino acid residues, at positions 13, 29, 30, and 79 (Smyth et al., 1963). These residues have not been implicated directly in catalysis, although three of them are in hydrophobic regions inside the protein (recent reviews: Barnard, 1969; Richards and Wyckoff, 1971; Moore and Stein, 1973). Alkylation or oxidation of the sulfur atom converts methionine from a hydrophobic residue (Nozaki and Tanford, 1963) to a charged hydrophilic one. Therefore, one can study the effects of changing the polarity of specific methionine residues on the structure and stability of the active conformation of ribonuclease. We have chosen alkylation, because at low pH this reaction is specific for methionine (Gundlach et al., 1959a,b; Stark et al., 1961). CH_3I was used as the alkylating reagent because the small size of the methyl group minimizes steric perturbation and because the product, a methyl sulfonium salt, is more stable than other alkyl or sulfoxide derivatives (Link and Stark, 1968). In a previous study, we showed that CH_3I modifies ribonuclease A completely and specifically on residue 29, with no effect on activity (Link and Stark, 1968; Link, 1970). Since methionine residues 13, 30, and 79 are not accessible to CH_3I without denaturation, the methylation reaction has now been studied in the presence of 8 *M* urea.

Experimental Procedures

Materials. These were as described previously (Link and Stark, 1968).

Assay. Enzymatic activity was assayed with 2′,3′-cyclic CMP (a gift of Dr. Merton Bernfield) according to Crook et al. (1960). The absorbancy at 291 nm of an 0.87 m*M* solution of the substrate increased from approximately 0.5 to 0.9 upon hydrolysis to 3′-CMP. Initial rates were determined by recording the first quarter of the reaction on a Cary 15 spectrophotometer equipped with a 0 to 0.1 absorbancy slide wire. One milliliter of freshly prepared 2′,3′-cyclic CMP stock solution was mixed with 1 ml of 0.2 *M* Tris-HCl buffer (pH 7.5) and enough 0.5 *M* KCl to make the total volume 2.5 ml after the addition of the enzyme, also in 0.5 *M* KCl. Each assay was repeated at three enzyme concentrations and the results were averaged.

Methylation Reactions in 8 M Urea. CH_3Met-29-RNase,[1] stored at 4° in 50% acetic acid, was prepared and transferred to 0.1 *M* KNO_3 by gel filtration on Sephadex G-25 as described by Link and Stark (1968). Peak fractions were combined and a reaction mixture was prepared in 10 ml, containing 0.1 *M* KNO_3, 0.1 *M* HCl, 8 *M* urea, and 0.22 m*M* CH_3Met-29-RNase (30 mg). The apparent pH was 2.8 at 25°. $^{14}CH_3I$ (0.10 mCi) was mixed with 0.25 ml of unlabeled CH_3I and added to initiate the reaction, which was performed in a stoppered vial at 25° in the dark. The heavier drop of CH_3I and the aqueous urea phase were mixed slowly by magnetic stirring. One-milliliter portions were removed at various times and added to 0.5 ml of 0.2 *M* L-cysteine to destroy the excess CH_3I. Samples were frozen immediately in ethanol–Dry Ice and stored at −20° until they could be desalted on a column (2.0 × 20 cm) of Sephadex G-25 equilibrated with 0.1 *M* KCl and 0.1 *M* Tris-HCl buffer (pH 7.0).

Protein concentration and content of methionine sulfone were determined by amino acid analysis of 0.05-mg samples after performic acid oxidation and hydrolysis as described by Link and Stark (1968). Samples of 1 to 2 mg were also chromatographed on a column (0.9 × 60 cm) of Amberlite

† From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305. *Received March 21, 1975.* This research was supported by grants from the National Institute of General Medical Sciences, National Institutes of Health, and from the National Science Foundation.

‡ Present address: Institute of Molecular Biology, University of Oregon, Eugene, Ore. 97403.

[1] Abbreviations used are: CH_3Met-29-RNase, CH_3Met-79-RNase, and CH_3Met-29-,CH_3Met-79-RNase, bovine pancreatic ribonuclease A methylated on methionine residues 29, 79, or 29 plus 79, respectively; $(CH_3Met)_4$-RNase, ribonuclease A methylated on all four methionine residues.

IRC-50 (XE-64) with 0.20 *M* sodium phosphate buffer (pH 6.30) at room temperature. ^{14}C was determined in duplicate as described by Link and Stark (1968). Transition temperatures were established by measuring the change in optical density at 237 mμ of 1 mg/ml solutions in the pH range 6.3 to 7.0 as a function of temperature as described by Link (1970).

The reaction of ribonuclease A with CH_3I in 8 *M* urea was performed as described above, except that the reaction was terminated by dilution and immediate gel filtration: 0.5-ml portions of the reaction mixture were diluted to 2.5 ml with 0.2 *M* acetic acid and desalted on a column (2.0 × 20 cm) of Sephadex G-25 in 0.2 *M* acetic acid. After adjusting the pH to 6.5, as much as 6 ml of protein solution was chromatographed on a column (0.9 × 60 cm) of IRC-50.

The fraction of enzymatic activity that would elute from IRC-50 was determined as follows. A reaction mixture in 8 *M* urea was prepared as above except that the protein concentration was 20 mg/ml. Samples (0.5 ml) were withdrawn at 10-min intervals at extents of reaction from 0 to 60% and diluted into 10 ml of 0.1 *M* glycylglycine buffer (pH 7.3) at 0° to stop the reaction. Half was chromatographed on a column (0.9 × 55 cm) of IRC-50. The first 500 ml of effluent, containing all the protein that will elute from this column, was pooled and prepared for assay by adjusting the pH to 7.5 and adding KCl to a final concentration of 0.25 *M*. The other half of each sample was diluted to 500 ml and adjusted similarly, but without chromatography. Each sample was then assayed at three enzyme concentrations as described above.

Preparation of Derivatives for Peptide Analysis. Ribonuclease A was methylated with $^{14}CH_3I$ in 8 *M* urea and desalted on Sephadex G-25 in 0.2 *M* acetic acid at 4° to stop the reaction. After adjusting the pH to 6.0, preparative chromatography was achieved on a 2.0 × 45 cm column of IRC-50 (Link and Stark, 1968). Separation of the active methylated derivatives from unreacted ribonuclease A is shown in Figure 3. These derivatives, along with $(^{14}CH_3)_4$-RNase prepared by exhaustive methylation (24 hr), were desalted into 50% acetic acid on Sephadex G-25 and taken to dryness with a rotary evaporator. Performic acid oxidation was performed according to Hirs (1956) and the proteins were separated from performic acid on a column (2.0 × 45 cm) of Sephadex G-25 in 0.1 *M* sodium phosphate buffer (pH 7.0) at 4°. Peak tubes were pooled and the samples were digested with α-chymotrypsin (Worthington 3× recrystallized) using a weight ratio of 1/200 (Hirs et al., 1956). After 24 hr, the digestion was terminated by adjusting the pH to 2.5 with HCl and the digest (2 to 10 mg) was fractionated by ion exchange chromatography.

Peptide Chromatography. Fifty grams of Aminex Q-155 resin (Bio-Rad) was prepared for use by washing successively with 250 ml each of 3 *M* NaOH, H_2O, 3 *M* HCl, and H_2O. The pH of an aqueous suspension of the resin was adjusted to 6.5 with pyridine and then to 2.50 by washing with formic acid. A 0.5 × 15 cm column was poured and washed with 300 ml of starting buffer. After each use the column was reequilibrated by stirring the top 5 cm with 5 ml of 60% pyridine and pumping starting buffer through until the pH of the effluent was 2.50.

Separation of the chymotryptic peptides of RNase was obtained using a linear gradient from pH 2.5 to 4.6, produced with a triple-chamber apparatus made according to the specifications for the two-chamber apparatus given by Hill and Delaney (1966). The pyridine–acetate volatile buffers of Schroeder et al. (1962) were used as described except for addition of formic acid to the starting buffer. The following buffers were placed in the first, second, and third chambers, respectively: (1) pH 2.50, 0.05 *M* pyridine: 500 ml of pH 3.10 buffer diluted to 2 l., pH adjusted with 42 ml of 88% formic acid; (2) pH 3.10, 0.20 *M* pyridine: 32.3 ml of pyridine adjusted to pH 3.10 with 557 ml of glacial acetic in 2 l.; (3) pH 4.60, 1.5 *M* pyridine: 232 ml of pyridine adjusted to pH 4.60 with 330 ml of glacial acetic acid in 2 l. The pyridine was purified by distillation from ninhydrin (Hill and Delaney, 1966). The column was operated to 50° using a flow rate of 30 ml/hr (20–50 psi) with 250 ml of each buffer. The effluent stream was divided, with 16 ml/hr to a fraction collector and the remainder to a Technicon Autoanalyzer. The alkaline hydrolysis and ninhydrin systems were operated as described by Hill and Delaney (1966), using the segmented method with no sample dilution prior to alkaline hydrolysis.

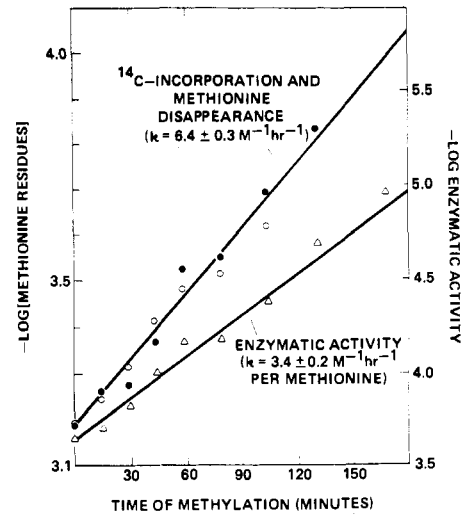

FIGURE 1: Semilog plot of changes in properties of CH_3Met-29-RNase with time of reaction in 8 *M* urea at 25°: (●) incorporation of ^{14}C into protein; (○) loss of methionine; (△) loss of activity.

Results

Methylation of CH_3Met-29-RNase in 8 M Urea. CH_3Met-29-RNase was prepared as described by Link and Stark (1968) by reaction of native ribonuclease A with CH_3I at low pH. It has the same enzymatic activity as RNase A (Link, 1970; see also Table II). The reaction of CH_3I with urea-denatured CH_3Met-29-RNase was followed using four independent measurements: (1) disappearance of methionine (determined as the sulfone after performic acid oxidation), (2) incorporation of ^{14}C from $^{14}CH_3I$, (3) decrease in enzymatic activity toward 2′,3′-cyclic CMP, and (4) chromatography on IRC-50. The time courses of incorporation of ^{14}C and disappearance of methionine both give the same pseudo-first-order rate constant for the methylation reaction (Figure 1), good evidence that only methionine is modified. The linearity of the plot indicates that all three methionines react with CH_3I at the same rate. Stark and Stein (1964) made the same observation for reaction in 8 *M* urea of the four methionine residues of ribonuclease A with iodoacetate and idoacetamide.

When the probability of reaction is the same at each site, the distribution of products can be calculated readily for any extent of the total reaction. (See Stark and Stein

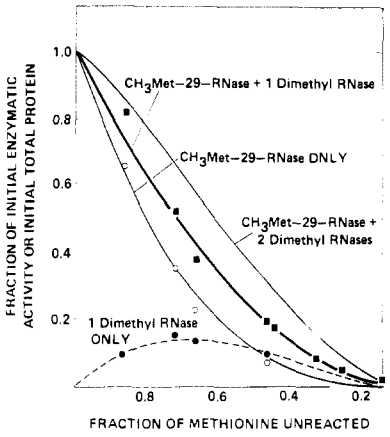

FIGURE 2: Correlation of changes in enzymatic activity and amounts of chromatographic peaks upon IRC-50 chromatography with the extent of methylation of the three methionines of CH_3Met-29-RNase in 8 *M* urea. The lines were calculated for several hypothetical possibilities, as noted in the figure, by assuming that the methionines are equally reactive: (■) residual enzymatic activity of reaction mixture as a function of extent of reaction (from ^{14}C incorporation); data from Figure 1; (○) fraction of total protein as unreacted CH_3Met-29-RNase (from IRC-50 chromatography); (●) fraction of total protein as a new peak at 1.7 times the elution volume of CH_3Met-29-RNase.

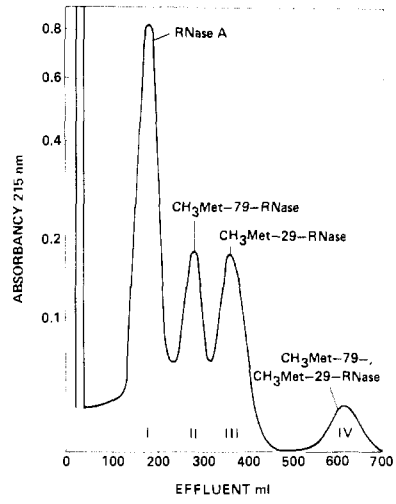

FIGURE 3: Preparative chromatography on IRC-50 of the products of reaction of CH_3I with ribonuclease A in 8 *M* urea. Protein (37 mg), methylated for 50 min to 24% completion, was loaded on the column. Elution was performed as described by Hirs et al. (1953).

Table I: Properties of the Proteins of Peaks II, III, and IV (Figure 3).

	Peak II	Peak III	Peak IV
Methionine residues methylated	79	29	29 and 79
Relative elution position (ribonuclease A = 1.0)	1.5	1.9	3.4
Relative enzymatic act. (ribonuclease A = 1.0)	0.9	1.1	0.9
Specific radioact. (cpm/nmol)	102	107	203
Free methionine (as the sulfone)	3.0	2.9	2.1

(1964) for such a correlation with the iodoacetate and iodoacetamide reactions.) The three solid curves in Figure 2 are the calculated amounts of unreacted CH_3Met-29-RNase remaining, plus either 1 or 2 equiv of dimethylated derivative. (Three different dimethyl derivatives can be produced from CH_3Met-29-RNase.) The broken line at the bottom of Figure 2 is the calculated fraction of protein present as a single dimethyl ribonuclease. The data for enzymatic activity and incorporation of ^{14}C from Figure 1 have been replotted as squares in Figure 2. These squares are near the curve calculated for the sum of unreacted CH_3Met-29-RNase and one dimethyl ribonuclease.

Portions of the reaction mixture taken at various times were chromatographed on IRC-50. A single new peak with two methionine residues methylated was eluted at 1.7 times the volume of CH_3Met-29-RNase, in the same position as peak IV of Figure 3. The amount of CH_3Met-29-RNase remaining unreacted, shown as open circles in Figure 2, is close to the calculated curve. Similarly, the amount of dimethyl ribonuclease formed, shown as closed circles in Figure 2, is the same as the amount of a single dimethyl protein calculated to be present (broken line). Finally, the fraction of the total protein present as the sum of the two IRC-50 peaks (open plus closed circles in Figure 2) is very similar to the fraction of the original enzymatic activity, determined before chromatography (squares in Figure 2), good evidence that only enzymatically active derivatives are eluted from IRC-50 under the conditions used.

Methylation of Ribonuclease A in 8 M Urea. Correlation of the extent of methylation with residual enzymatic activity (as in Figure 2) did not provide enough information to establish the number of active derivatives in the more complicated situation of ribonuclease A, where four different monomethyl and six different dimethyl derivatives are produced. Experiments similar to the one shown in Figure 2 established that all four methionines are methylated at the same rate in 8 *M* urea, 25° (see Stark and Stein, 1964). Chromatography on IRC-50 of reaction mixtures after removal of the urea revealed three methylated derivatives in addition to unreacted ribonuclease A (Figure 3). Peaks II and III are monomethylated by amino acid analysis for methionine and content of ^{14}C relative to $(^{14}CH_3Met)_4$-RNase, and peak IV is a dimethyl ribonuclease by these criteria (Table I). Peaks III and IV are eluted from IRC-50 in the same positions as CH_3Met-29-RNase and its only active methylation product, respectively. All three peaks have the same specific enzymatic activity as ribonuclease A.

The percentages of the initial protein found in peaks I, II, and III, determined for several extents of reaction, are shown in Table II. These data further indicate that peaks II and III are each a single monomethyl derivative of ribonuclease A. Only three active derivatives of ribonuclease A are eluted from IRC-50. To decide whether there are other active derivatives that do not elute from this resin, another experiment was performed. The enzymatic activities eluted were compared to the activities of duplicate unchromatographed samples, diluted to the same volume, as a function of the extent of methylation. The average value is 89.8 ± 3.5% (seven determinations, 5–60% reaction). The recovery for unreacted ribonuclease A, chromatographed or diluted, is 91.0 ± 1.2% for five determinations. A single fully active monomethyl derivative that failed to elute from IRC-50 would reduce the total activity recovered by about 8% (averaging from 5 to 60% reaction). Therefore, all the enzymatic activity formed after renaturation of ribonuclease–CH_3I reaction mixtures from urea is eluted from IRC-50 in four peaks. An interesting corollary is that none of the 12 possible inactive derivatives are eluted.

Table II: Recovery of Monomethyl Ribonuclease from IRC-50.[a]

Reaction Time (min)	Percentage of Total Protein					
	Peak I (Ribonuclease A)		Peak II (CH_3Met-79-RNase)		Peak III (CH_3Met-29-RNase)	
	Found	Calcd	Found	Calcd	Found	Calcd
0	=100	100	0	0	0	0
15	67	57	12	9	12	9
30	36	36	13	11	12	11
45	22	22	13	10	13	10
60	11	14	11	9	7	9

[a] Portions of a single reaction mixture were chromatographed as described in the text. The amount of protein found in each peak is expressed as a percentage of the amount of ribonuclease A found at zero time. The calculated amounts are established from the rate constants determined as in Figure 1 for the methylation and the fact that all four methionines react at the same rate. If the fraction of total methionine unreacted is X, the fraction of ribonuclease remaining is X^4 and the fraction of a single monomethyl derivative is $X^3(1-X)$.

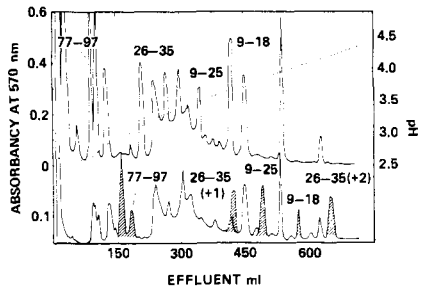

FIGURE 4: Chromatography of peptides derived from 8.1 mg of ribonuclease A (upper curve) or 6 mg of ($^{14}CH_3$Met)$_4$-RNase (lower curve) after oxidation and chymotryptic digestion. See the text for details. The numbers refer to the residues included in each peptide and are given only for those peptides which contain methionine. Peptides containing radioactivity ($^{14}CH_3$-methionine) are shaded. The shaded peptide 26–35 (+1) is obtained only from CH_3Met-29-RNase and CH_3Met-29-,CH_3Met-79-RNase; it contains only one methyl group (at position 29) but two methionines (at positions 29 and 30). The dashed line gives the pH.

Separation of Peptides Containing Methionine and S-Methylmethionine. The peptides resulting from an α-chymotryptic digest of oxidized ribonuclease separate well on Aminex Q-155 resin using a linear gradient of salt and pH. The upper and lower curves of Figure 4 are the patterns for the peptides from ribonuclease A and ($^{14}CH_3$Met)$_4$-RNase, respectively. The peptides containing methionine are marked with the residue numbers of the constituent amino acids, from the sequence of Smyth et al. (1963), and the peaks containing [*methyl*-^{14}C]methionine are shaded. Amino acid analyses of these peaks are presented elsewhere (Link, 1970). Methylation of each methionine increases the net positive charge of the peptide by +1 and the increased positive charge retards elution from the negatively charged resin by 0.35 to 0.39 pH unit for the peptides containing Met-13 or Met-79. Met-29 and Met-30 are in the same peptide (26 to 35) and methylation retards elution by 0.58 pH unit for each site alkylated.

Met-13 is found in two peptides due to a partial chymotryptic cleavage between Ser-18 and Ala-19. Since α-chymotrypsin prefers hydrophobic side chains it is not surprising that methylation of Met-79 prevents the cleavage between CH_3Met-79 and Ser-80. The tripeptide 77 to 79 remains attached to residues 80 to 97. Peptide 77 to 97 from ($^{14}CH_3$Met)$_4$-RNase and peptide 80 to 97 from ribonuclease A separate for unknown reasons into two peaks with the same amino acid compositions.

Table III: Properties of Labeled Chymotryptic Peptides Obtained from Digests of Peaks II, III, and IV (Figure 3) after Performic Acid Oxidation.

	Peak II	Peak III	Peak IV	
Residue no.'s of labeled peptides	77–97	26–35	77–97	26–35
pH of elution	2.97–3.02	3.68	2.88–3.00	3.69
Recovery of ^{14}C (%)	35	59	40	60
Amino Acids in Labeled Peptides	Residues/Peptide[a]			
Asp	1.8 (2)[b]	2.1 (2)	2.4 (2)	1.8 (2)
Thr	2.5 (3)	0.3	2.2 (3)	0.3
Ser	3.7 (4)	1.2 (1)	4.0[c] (4)	1.4 (1)
Glu	1.0[c] (1)	1.1 (1)	1.8 (1)	1.1 (1)
Pro	1.3 (1)	0	*d* (1)	*d*
Gly	1.0 (1)	0.2	1.8 (1)	0.1
Ala	0.9 (1)	0.2	1.7 (1)	0.2
$CysSO_3$	0	0.8 (1)	0	0.7 (1)
Val	0	0	0.7	0
$MetSO_2$	0	0.8 (1)	0	0.9 (1)
Ile	0.7 (1)	0	0.7 (1)	0
Leu	0	1.0[c] (1)	0.3	0.8 (1)
Tyr	0.9 (2)	0	*d* (2)	*d*
Phe	0	0	*d*	*d*
Lys	1.7 (1)	1.3 (1)	*d* (1)	*d* (1)
His	0.3	0.2	*d*	*d*
Arg	1.0[c] (1)	1.0[c] (1)	*d* (1)	*d* (1)

[a] Hydrolyses were performed as described by Link and Stark (1968). No correction was made for destruction during hydrolysis. Residues are rounded to nearest 0.1. [b] Numbers in parentheses are theoretical numbers of residues. [c] Basis of calculation of residues/peptide. [d] Not determined.

Sites of Methylation. The $^{14}CH_3$-labeled peaks II, III, and IV from the chromatogram shown in Figure 3 were desalted, oxidized, and digested with α-chymotrypsin and the resulting peptides were fractionated as in Figure 4. Sites of methylation were established from the elution positions and amino acid compositions of the radioactive peptides. Data for the labeled peptides are shown in Table III. The protein from peak II is methylated on Met-79 and that from peak III is methylated on Met-29. The protein from peak IV yields the same radioactive peptides as those obtained from both peaks II and III and must be modified on Met-79 and Met-29. The conclusion that the protein of peak III is methylated on Met-29 and not Met-30 is indirect, because Met-29 and Met-30 are in the same chymotryptic peptide, but the argument is a strong one. Link and Stark (1968) showed that the only derivative resulting from the methylation of undenatured ribonuclease was modified on Met-29. CH_3Met-29-RNase and the protein in peak III elute from IRC-50 at the same position and have the same melting temperature (see the Discussion). As noted in Table II, the amount of peak III is the same as that of peak II (modified on Met-79) and therefore peak III is not a mixture of derivatives modified singly on Met-29 or Met-30 which fails to separate.

Discussion

Refolding and Stability of Methylmethionine Derivatives. Met-29 reacts readily with CH_3I in undenatured ribo-

nuclease A, at about half the rate of *N*-acetylmethionine (Link and Stark, 1968), implying that the sulfur atom is exposed at least partially to the solvent in the native structure. Conversely, the properties of CH_3Met-29-RNase are very similar to those of ribonuclease A. The melting temperature is 58°, only 5° lower than that of ribonuclease A under the same conditions, and the specific activity toward 2′,3′-cyclic CMP is indistinguishable from that of ribonuclease A. In a more extensive study of the properties of this derivative, Link (1970) has shown that the pH dependence of the activity toward 2′,3′-cyclic CMP is identical with that of ribonuclease A over the entire pH range 4.0–8.5. However, ribonuclease A is 2.6 times more active toward *Escherichia coli* tRNA at pH 7.5. The specific and facile methylation of Met-29, with minimal effect on the properties of the enzyme, suggests that Met-29 may be an excellent site for introducing conformational probes by chemical modification.

Methylation of Met-79 causes much greater destabilization. For comparison, the melting temperatures of several derivatives are listed below, including ribonuclease S and CH_3Met-29-RNase S, prepared and studied by Link (1970): ribonuclease A, 63°; CH_3Met-29-RNase, 58°; CH_3Met-79-RNase, 43°; ribonuclease S, 42°; CH_3Met-29-RNase S, 39°; CH_3Met-29-,CH_3Met-79-RNase, 36°. Each of three different alterations of ribonuclease A (methylation of Met-29 or Met-79 or cleavage of the peptide bond between Ala-20 and Ser-21) causes destabilization of the structure, but each of the derivatives retains full activity. It appears that the destabilizing effects of two modifications within the same molecule are roughly additive.

Since all of the enzymatic activity present after renaturation of reaction mixtures of ribonuclease and CH_3I in 8 *M* urea can be recovered as unmodified ribonuclease A plus the three derivatives modified on Met-29 and Met-79, it is clear that no derivative methylated on Met-13 or Met-30 can refold to an active conformation under the conditions employed (IRC-50 chromatography or dilution into 0.2 *M* sodium phosphate (pH 7.5), 25°). The success of our approach is based on the fact that all 12 inactive derivatives do not elute from IRC-50. This result contrasts with the results obtained following modification of the methionine residues of ribonuclease A at pH 2.8 and 40° with iodoacetate (Gundlach et al., 1959b) and with H_2O_2 (Neumann et al., 1962). In both these instances, multiple peaks of inactive protein were eluted from IRC-50 under conditions essentially the same as those we have used.

Methionine-29 and Methionine-79. From the structural data of Richards and Wyckoff (1971) on crystals of ribonuclease S at 2.0-Å resolution, Met-29 is the only methionine residue with its sulfur atom partly exposed to the solvent, consistent with the results of Link and Stark (1968). Met-79 is definitely inside the molecule, with little or no accessibility to solvent. Methylation of ribonuclease A at Met-79 causes a decrease in melting temperature of 20°, perhaps due to some disruption of the structure required to give the charged sulfonium salt access to solvent. Met-29 is in a region of α-helix in both ribonuclease A (Carlisle et al., 1974) and ribonuclease S (Wyckoff et al., 1970). The optical rotatory dispersion spectrum of CH_3Met-29-RNase is indistinguishable from that of ribonuclease A (Link, 1970), indicating that the helix content is not altered substantially by methylation of Met-29. Link (1970) has shown that the ultraviolet spectrum and the pH dependence of tyrosine ionization are also identical for CH_3Met-29-RNase and ribonuclease A.

Methionine-13 and Methionine-30. From the crystal structure of ribonuclease S, Met-13 and Met-30 are among a group of about 13 residues comprising a hydrophobic core (Richards and Wyckoff, 1971). Goren and Barnard (1970a,b) have prepared a fully active derivative of ribonuclease A by carboxymethylation with bromoacetate at pH 5.5 and have found that the site of modification is Met-30. This result is unexpected, considering the hydrophobic environment of this residue and the evidence presented here that methylation of Met-30 in urea prevents refolding to an active conformation. Goren and Barnard conclude that, since this reaction is inhibited by 3′-CMP, modification occurs by means of access through the active site. The effect of this inhibitor in the carboxymethylation reaction is in contrast to the finding of Link and Stark (1968) that methylation of native ribonuclease A at Met-29 is not inhibited by active-site ligands. Possibly the active carboxymethyl derivative, like CH_3Met-30-RNase, might not refold from 8 *M* urea and can only be formed if the native structure remains undisrupted. It is not clear why the exposed sulfur atom of Met-29 fails to react with bromoacetate.

The effects of alkylating Met-13 in ribonucleases A and S are very different, but can be rationalized by comparing the crystal structures of the two enzyme forms. Because Met-13 is present in S-peptide (residues 1–20) it has been studied extensively. (See the review of Richards and Wyckoff (1971) for an extensive discussion of the work of Richards and coworkers with modifications of S-peptide and the work of Scoffone and Hofmann and their coworkers with synthetic analogs.) Thirty times more of peptide 1–12 than of peptide 1–13 must be added to S-protein (residues 21–124) to generate the same activity, and 10 times more of the sulfoxide of peptide 1–13 is required than unmodified 1–13, which itself is one-sixth as effective as S-peptide. Oxidation of Met-13 to the sulfone in S-peptide increases by 40 times the molar amount required to give 50% activity and 150 times more carboxymethyl-Met-13 or carboxyamidomethyl-Met-13-S-peptide are required than S-peptide. From these results, it is clear that Met-13 is not essential for activity, although it is important for binding to S-protein. Why then should methylation of Met-13 in ribonuclease A cause an inability to renature to an active conformation? With the peptide bond 20–21 still intact, binding of the S-peptide region of ribonuclease A to the rest of the protein should be tight enough to bring His-12 into the active site, even if the contribution of Met-13 to hydrophobic stabilization has been lost. In the crystal structure of ribonuclease S, Richards and Wyckoff (1971) report that rotation of the side chain of Met-13 about the α-β carbon–carbon bond would allow the charged sulfur atom of this residue access to the solvent without any major change in the conformation of S-peptide. In a recent X-ray crystal structure of ribonuclease A, Carlisle et al. (1974) note that the constraint forced on the unbroken peptide chain in ribonuclease A has the effect of reducing α-helix content between Thr-3 and Met-13, and that the splitting of the chain between Ala-20 and Ser-21 in ribonuclease S has allowed the residues in this region more freedom of movement. In ribonuclease A, Met-13 borders the hydrophobic region found at the deep end of the active-site cleft and is protected from the exterior by Leu-15. Therefore, in ribonuclease A, it seems likely that a sulfonium derivative of Met-13 could not gain access to the solvent without a large change in the structure.

Oxidation of Met-29 and Met-13 in ribonuclease A has

consequences similar to methylation. In a study of the reactivity of methionine in ribonuclease A to photooxidation, Jori et al. (1970) found that only Met-29 was oxidized in concentrations of acetic acid up to 10%. This derivative is enzymatically and conformationally similar to ribonuclease A, as is CH_3Met-29-RNase. Between 10 and 50% acetic acid, Met-13 is also oxidized. The disulfoxide has low activity, in contrast to the high activity of S-protein recombined with oxidized S-peptide (Vithayathil and Richards, 1960).

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