Hinson, J. A., and Neal, R. A. (1972), *J. Biol. Chem. 247*, 7106.
Lee, J. (1972), *Biochemistry 11*, 3350.
Lee, J., Wesley, A. S., Ferguson, J. F., and Seliger, H. H. (1966), *in* Bioluminescence in Progress, Johnson, F. H., and Haneda, Y., Ed., Princeton, N. J., Princeton University Press, p 35.
McCapra, F., and Hysert, D. W. (1973), *Biochem. Biophys. Res. Commun. 52*, 298.
McElroy, W. D., and Green, A. A. (1955), *Arch. Biochem. Biophys. 56*, 240.
Michaliszyn, G. A., *et al.* (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32*, 627.
Mitchell, G., and Hastings, J. W. (1971), *Anal. Biochem. 39*, 243.
Shimomura, O., Johnson, F. H., and Kohama, Y. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2086.
Strehler, B. L., Harvey, E. N., Chang, J. J., and Cormier, M. J. (1954), *Proc. Nat. Acad. Sci. U. S. 40*, 10.
Sund, H., and Theorell, H. (1963), *Enzymes, 2nd Ed. 7*, 25.

Spin-Labeled Ribonuclease A. Selective Incorporation of a Nitroxide Spin Label Sensitive to Active-Center Geometry†

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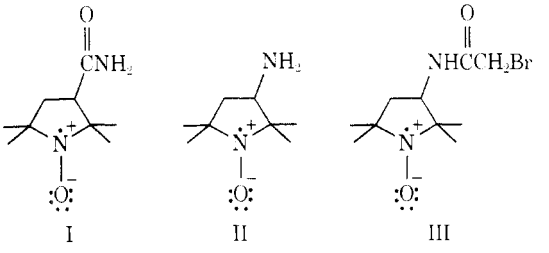
ABSTRACT: The reaction of *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)bromoacetamide with bovine pancreatic ribonuclease A has been investigated at pH 5.50. The two principle spin-labeled products, 3-SLHis-105-RNase A and a *S*-SLMet-RNase A, are slowly formed indicating that no special rate enhancement effects such as electrostatic positioning of the spin label for reaction, etc., are manifest. Furthermore, under the conditions used, no spin labeling occurs at histidine-12 or histidine-119. The site of alkylation in the 3-SLHis-105-RNase A derivative has been assigned to the number three imidazole nitrogen of histidine-105 by application of the subtractive Edman degradative procedure (one step) to a carboxyl-terminal tryptic peptide (residues 105–124) which was isolated. The 3-SLHis-105-RNase A has a specific enzymatic activity of ~85% that of native RNase A with both 2′,3′-cyclic cytidylic acid and yeast tRNA as substrates and exhibits active-site chemistry identical with the native enzyme. The evidence suggests that the covalently attached spin label is sensitive to active-center geometry and may therefore be useful in elucidating the conformational properties of the molecule.

Of the many methods utilized in investigating the structure of macromolecules in solution, those involving magnetic resonance have proved valuable. One of these, the spin-label technique (Stone *et al.*, 1965; Hamilton and McConnell, 1968; Griffith and Waggoner, 1969; McConnell and McFarland, 1970), has been used frequently because of its applicability to the study of a variety of biological systems. An investigation of bovine pancreatic ribonuclease A by the spin-label approach has been undertaken in this laboratory for several reasons. The primary sequence of RNase A is known (Smyth *et al.*, 1963), the tertiary structure of the molecule has been elucidated by X-ray diffraction (Kartha *et al.*, 1967), and many enlightening chemical and physical modifications of the enzyme have been accomplished (Barnard, 1969). In short, because of the vast amount of information already available concerning the enzyme, a study of RNase A by the spin-label method should provide highly interpretable results.

Shortly after our studies were initiated, Smith (1968) reported that the bromoacetamide nitroxide spin label III,



along with several other spin labels, had been employed to investigate conformational aspects of RNase A. In his studies, however, Smith dealt with the total reaction mixtures and did not separate these mixtures or attempt to identify the components; accordingly, the conclusions reached from these studies were considered preliminary. The present communication describes our investigation of the reaction of the spin label III with RNase A at pH 5.50 and the isolation and characterization of a ribonuclease A derivative spin labeled at histidine.

Experimental Section

Materials. Bovine pancreatic ribonuclease A (EC 2.7.7.16) (type IIA), trypsin (EC 3.4.4.4) (dicycohexylcarbodiimide-treated type XI), and cyclic 2′,3′-cytidylic acid, sodium salt, were purchased from Sigma Chemical Co., yeast tRNA (lot 6602) was obtained from Schwarz BioResearch, Inc., and an affinity resin for ribonuclease A [Sepharose coupled with 5′-

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(4-aminophenylphosphoryl)uridine 2′(3′)-phosphate] can be purchased from the Research Division of Miles Laboratories in Kankakee, Ill. The Sigma RNase A was further purified by the chromatographic procedure of Crestfield *et al.* (1963b) and desalted on Sephadex G-25. 3-Carboxymethylhistidine was prepared by the procedure of Crestfield *et al.* (1963c).

N-(1-Oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)bromoacetamide (III). The nitroxide I (2,2,5,5-tetramethyl-3-carbamidopyrrolidine-1-oxyl) was converted to 2,2,5,5-tetramethyl-3-aminopyrrolidine-1-oxyl (II) as described by Rozantzev and Krinitzkaya (1965). The crude amine, recovered as an orange, paramagnetic oil by ethereal extraction and rotory evaporation, was used directly to form the bromoacetamide (III) without further purification (Morrisett, 1968).

Methods. Absorbance measurements were carried out with a Unicam SP1800 ultraviolet spectrometer and electron paramagnetic resonance (epr) spectra were recorded at 25° using a Joelco JES-ME-3X resonance spectrometer at 9.5 GHz (X band) with 100-kHz modulation. Samples for amino acid analysis were hydrolyzed with constant-boiling HCl (bp 108°) *in vacuo* at 110° for 24 hr. Amino acid analyses were carried out on a Phoenix Model VG6000B amino acid analyzer which had been modified to a two-column system. Residue numbers were based wherever possible on glycine and alanine for the acidic and neutral acids and on arginine for the basic amino acids.

Assay of Ribonuclease A. Enzymatic activity toward 2′,3′-cyclic cytidylic acid as substrate was determined by the spectrophotometric method of Crook *et al.* (1960). The substrate concentration used was 0.11 mg/ml in 0.1 M Tris-HCl buffer containing 0.1 M NaCl (pH 7.00). Activities using yeast tRNA as substrate were determined by the method of Kunitz (1946). Protein concentrations, in the case of RNase A and its spin-labeled derivatives, were determined spectrophotometrically at 278 nm using 9800 as the molar extinction coefficient (Hermans and Scheraga, 1961).

Reaction of Ribonuclease A with the Bromoacetamide Nitroxide Spin Label, III. In a typical spin-labeling reaction, 450 mg (33 μmol) of purified RNase A was dissolved in 45 ml of 0.1 N sodium acetate buffer (pH 5.50). To this solution was added 150 mg (540 μmol) of the bromoacetamide nitroxide spin label, III, in small portions with vigorous stirring. In about 10 min, all of the spin label was dissolved and the pH was readjusted to 5.50 if necessary. The reaction was allowed to proceed with stirring in the dark at 25° for 72 hr. The entire reaction mixture was then applied to a column (3.8 × 50 cm) of Sephadex G-25 equilibrated with 5% acetic acid and chromatographed. The chromatography served to desalt the reaction mixture as well as to remove the excess unreacted spin label III, thus terminating the reaction. The effluent was monitored at 280 nm and the fractions corresponding to the desalted reaction mixture were pooled, lyophilized, and stored at −20°.

Preparation of the Products of the Spin-Label Reaction. The desalted reaction mixture was dissolved in 4 ml of 0.2 M sodium phosphate buffer (pH 6.47) and chromatographed at a flow rate of 15–20 ml/hr on a column (2.5 × 135 cm) of Bio-Rex 70 (−400 mesh). Three major peaks absorbing at 280 nm emerged as shown in Figure 1. The fractions comprising each of the three peaks were pooled and respectively lyophilized. Each peak was then desalted by chromatography on Sephadex G-25 equilibrated in 5% acetic acid and the resulting protein containing fractions were lyophilized. Amino acid analyses were carried out directly on the isolated products (see Table I).

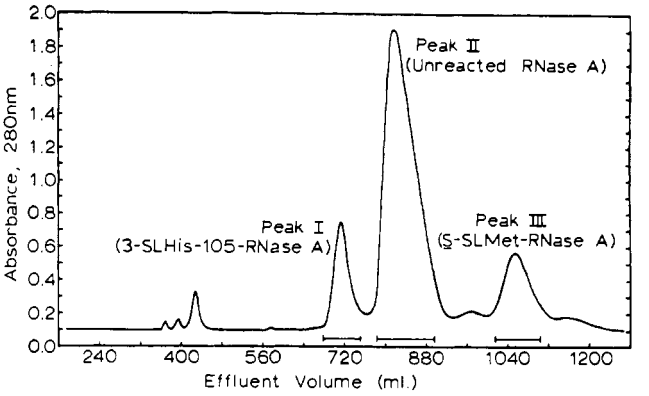

FIGURE 1: Chromatographic separation of components of spin-label reaction mixture on Bio-Rex 70 in 0.2 M sodium phosphate (pH 6.47): ribonuclease A + 16.4-fold molar excess of the bromoacetamide nitroxide spin label III, 0.1 N sodium acetate (pH 5.50), for 3 days.

Peak III material undergoing this desalting procedure exhibited an epr spectrum with an appreciable amount of mobile component (as in Figure 2d) while the epr spectrum of peak III in 0.2 M sodium phosphate from the chromatography showed none. An alternative procedure involving ultrafiltration using an Amicon UM-2 filter, Bio-Rex 70 NaCl chromatography (Crestfield, 1963), and then immediate concentration by ultrafiltration enabled the epr spectrum in Figure 2c to be recorded.

TABLE I: Amino Acid Composition of Components of Spin-Label Reaction.

	No. of Amino Acid Residues/Molecule[a]			
Amino Acid	RNase A	Peak I 3-SLHis-105-RNase A	Peak II Unreacted RNase A	Peak III *S*-SLMet-RNase A
Aspartic acid	14.9	15.2	15.0	14.9
Threonine[c]	9.43	9.46	9.56	9.53
Serine[c]	12.7	13.4	12.8	12.9
Glutamic acid	12.1	12.0	12.1	12.0
Proline	4.08	4.03	4.06	3.98
Glycine	3.02	3.01	3.05	3.54
Alanine	12.0	12.0	11.9	12.0
Cystine[c]	7.19	8.18[d]	6.95	7.24
Valine	8.92	8.56	9.02	8.90
Methionine	4.01	3.65	3.66	3.20[e]
Isoleucine[b]	2.41	2.02	2.46	2.38
Leucine	2.07	1.92	2.07	2.11
Tyrosine[c]	5.78	5.12	5.66	5.40
Phenylalanine	3.02	2.92	3.07	2.96
Lysine	9.90	10.2	10.1	10.1
Histidine	4.00	2.97	3.96	4.00
Arginine	4.00	4.00	4.04	4.00

[a] Each column represents the average of the results of two 24-hr hydrolysates. [b] The isoleucylisoleucine sequence is incompletely hydrolyzed in 24 hr. [c] Not corrected for decomposition during acid hydrolysis. [d] Includes 3-carboxymethylhistidine. [e] *S*-Carboxymethylhomocysteine and homoserine lactone were present in the spectrum but were not quantitated. The value for methionine is uncorrected.

TABLE II: Amino Acid Composition of C-Peptide Isolated from Peak I (3-SLHis-105-RNase A).

Amino Acid	No. of Amino Acid Residues/Molecule: C-Peptide[a] from Peak I (3-SLHis-105-RNase A)	Theory
Aspartic acid[b]	0.22	
Threonine	1.02	1
Serine[b]	0.28	
Glutamic acid[c]	3.38	3
Alanine	3.06	3
3-Carboxymethylhistidine	0.00	0
Phenylalanine	1.00	1
Homoserine lactone + homoserine	*d*	1
Lysine	1.98	2
Histidine	0.99	1
Arginine	1.02	1

[a] This column represents the average of the results of two 24-hr hydrolysates. [b] These values arise from a small amount of C-protein (less than 2% based on the mole concept). [c] This value is high due to the presence of a small amount of C-protein and homoserine. [d] Both of these amino acid derivatives were present but not quantitated.

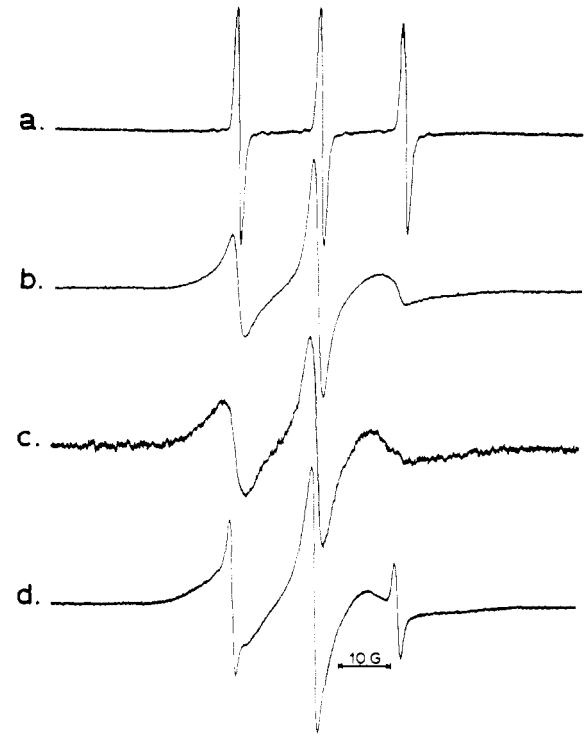

FIGURE 2: Electron paramagnetic resonance spectra of (a) free bromoacetamide nitroxide spin label III in water, (b) 3-SLHis-105-RNase A in 0.1 N sodium acetate (pH 5.50), (c) *S*-SLMet-RNase A (Peak III) in 0.226 *m* sodium chloride, (d) *S*-SLMet-RNase A (peak III) in water after appreciable denaturation.

Cyanogen Bromide Cleavage of 3-SLHis-ribonuclease A.[1] The procedure of Gross and Witkop (1962) was followed. The amino-terminal tridecapeptide (C-peptide) was isolated by Sephadex G-25 chromatography (1.9 × 130 cm column). To render it sufficiently free of the previously eluting C-protein a rechromatography of the C-peptide fraction was necessary (Table II).

Location of the Spin-Labeled Histidine Residue in 3-SLHis-ribonuclease A. Paralleling the approach followed by Crestfield *et al.* (1963c), 30 mg of the 3-SLHis-RNase A was reduced with β-mercaptoethanol and the resulting cysteine sulfhydryl groups were carboxymethylated with iodoacetic acid (Crestfield *et al.*, 1963a). Approximately 2 mg of the RCM-3-SLHis-RNase A was subjected to amino acid analysis (see Table III). The reduced, carboxymethylated material was then digested with trypsin for 8 hr (Crestfield *et al.*, 1963a) and the resulting mixture desalted as previously described (Crestfield *et al.*, 1963c). From the salt-free peptide mixture, the carboxyl-terminal eicosapeptide (residues 105–124), hereafter designated RCM-Trp-16, was isolated by gradient chromatography on the acid form of Bio-Rex 70 (−400 mesh) as described by Crestfield *et al.* (1963c). The fractions containing the RCM-Trp-16 peak, emerging at ~60 ml, were pooled. Half of the RCM-Trp-16 solution was concentrated to dryness and submitted to amino acid analysis (see Table IV). The remaining half was concentrated to dryness and the residue subjected to one stage of the Edman degradation by the procedure of Konigsberg and Hill (1962). Acid hydrolysis and amino acid analyses (Table IV) were carried out directly on completion of the fragmentation step without purification of the degraded peptide.

Results

When the bromoacetamide nitroxide spin label III reacted with ribonuclease A at pH 5.50 and the reaction mixture was separated by chromatography, three major peaks emerged (Figure 1). Amino acid analyses performed on the three peaks are shown in Table I.

The major constituent of the reaction mixture, peak II, has an elution position in the chromatography identical to that for unreacted native ribonuclease A. The material gives amino acid analysis data identical to that for native RNase A and exhibits full enzymatic activity toward yeast tRNA and 2′,3′-cyclic cytidylic acid. Analysis of the composition of peak II by chromatography on the Bio-Rex 70 NaCl analytical system of Crestfield (1963) resulted in >90% of the material eluting as a spin-negative, symmetrical peak at a position where native RNase A is normally observed. The simplest explanation is that peak II is unreacted RNase A contaminated by a small amount (<10%) of a spin-labeled derivative not sufficient to affect the amino acid analysis outcome.

The amino acid analysis data for peak III indicates that a methionine residue has been spin labeled. This conclusion is evident by the low integration for methionine (3.2 residues instead of the theoretical 4) coupled with the appearance in the amino acid analysis spectrum of *S*-carboxymethylhomocysteine and homoserine lactone which are breakdown products known to occur when *S*-carboxymethylmethioninesulfonium salts are acid hydrolyzed (Gundlach *et al.*, 1959a).

The epr spectrum of Bio-Rex 70 NaCl-purified *S*-SLMet-RNase A (peak III) in 0.266 *m* NaCl is shown in Figure 2c.

[1] Abbreviations used are: CM, carboxymethyl; RCM, reduced carboxymethyl; SL, spin-labeled; RCM-Trp-16, peptide (residues 105–124) assigned by the nomenclature of Hirs *et al.* (1956) which results from treatment of reduced and carboxymethylated 3-SLHis-RNase A with the enzyme trypsin.

TABLE III: Amino Acid Composition of Reduced, Carboxymethylated Peak I (RCM-3-SLHis-105-RNase A).

Amino Acid	No. of Amino Acid Residues/Molecule: RCM-Peak I[a,b] (RCM-3-SLHis-105-RNase A)	Theory
S-Carboxymethylcysteine	8.56	8
Aspartic acid	14.8	15
Threonine[d]	9.45	10
Serine[d]	12.9	15
Glutamic acid	12.1	12
Proline	3.95	4
Glycine	3.10	3
Alanine	11.9	12
3-Carboxymethylhistidine	0.95	1
Valine	8.85	9
Methionine	3.89	4
Isoleucine[c]	2.26	3
Leucine	2.04	2
Tyrosine[d]	5.58	6
Phenylalanine	3.03	3
Lysine	9.65	10
Histidine	3.08	3
Arginine	3.92	4

[a] This column represents the average of the results of two 24-hr hydrolysates. [b] RCM denotes reduced, carboxymethylated. [c] The isoleucylisoleucine sequence is incompletely hydrolyzed in 24 hr. [d] Not corrected for decomposition during acid hydrolysis.

TABLE IV: Amino Acid Composition of RCM-Trp-16 Isolated from Peak I (3-SLHis-105-RNase A).

Amino Acid	RCM-Trp-16, from 3-SLHis-105-RNase A (Residues 105–124)[a,b]: As Isolated Found 1	As Isolated Theory 2	After Edman Degradation Found 3	After Edman Degradation Theory 4
S-Carboxymethylcysteine	1.04	1	1.06	1
Aspartic acid	2.02	2	2.02	2
Serine[d]	0.86	1	0.86	1
Glutamic acid	1.08	1	1.08	1
Proline	2.01	2	2.07	2
Glycine	1.02	1	1.04	1
Alanine	1.98	2	1.96	2
3-Carboxymethylhistidine	1.00	1	0.44	0
Valine	3.86	4	3.82	4
Isoleucine[c]	1.42	2	1.51	2
Leucine	0.05		0.05	
Tyrosine[d]	0.91	1	0.91	1
Phenylalanine	1.00	1	1.00	1
Lysine	0.08		0.10	
Histidine	1.08	1	1.06	1

[a] Columns 1 and 3 represent the results of a single 24-hr hydrolysis. [b] RCM denotes reduced, carboxymethylated. [c] The isoleucylisoleucine sequence is incompletely hydrolyzed in 24 hr. [d] Not corrected for decomposition during acid hydrolysis.

A slow, irreversible denaturation of the molecule occurs with time giving rise to a steadily growing mobile component in the epr spectrum (Figure 2d). The specific activity of the undenatured derivative was found to be 100 ± 3% with 2′,3′-cyclic cytidylic acid as substrate. Due to its inherent lack of stability, the peak III material has not been further investigated.

On comparison of the amino acid analysis data for peak I with that for native ribonuclease A (Table I), two differences are immediately obvious. The number of residues of histidine has decreased from four to three and the value for half-cystine is higher than that normally observed for native RNase A. Since 3-carboxymethylhistidine is known to coelute with cystine on the long column of the amino acid analyzer (Crestfield *et al.*, 1963c), the differences mentioned above indicated that one residue to histidine per molecule of spin-labeled enzyme has been modified and that the spin label is attached by a covalent bond to the nitrogen in the 3 position of the imidazole ring of that histidine. In the amino acid analysis spectrum, no peaks in the elution positions of 1-carboxymethylhistidine, dicarboxymethylhistidine, or any other carboxymethylamino acid derivative (excepting 3-carboxymethylhistidine) could be detected.

To determine if the spin label was attached to the histidine-12, a cyanogen bromide cleavage was carried out on the peak I material with subsequent isolation of the C-peptide fragment (residues 1–13 in sequence) by gel filtration chromatography. The amino acid analysis for this amino-terminal peptide is shown in Table II. The peptide contains no 3-carboxymethylhistidine but rather a single unmodified histidine residue as would be expected if the spin label is attached to one of the other three histidines (at positions 48, 105, and 119 in sequence) of RNase A.

To distinguish which of the remaining three histidines was spin labeled, the carboxyl-terminal 105 to 124 amino acid peptide (RCM-Trp-16) was isolated from a trypsin digest of the reduced and carboxymethylated peak I material. (Table III shows the amino acid analysis data of reduced and carboxymethylated peak I. With the half-cystines converted to *S*-carboxymethylcysteines, the 3-carboxymethylhistidine was no longer obscured and could be quantitated.) Amino acid analysis of the isolated RCM-Trp-16 (column 1, Table IV) demonstrates that the carboxyl-terminal peptide was pure and contained 100 ± 5% of the 3-carboxymethylhistidine. Since histidine-105 was amino-terminal in the peptide, a distinction could be achieved by subjecting the RCM-Trp-16 to one stage of the Edman degradation (Konigsberg and Hill, 1962). Examination of the amino acid analysis data of the resulting peptide (column 3, Table IV) shows that the 3-carboxymethylhistidine has been removed in ~56% yield while the observed value for unmodified histidine remains unchanged. It would follow then that the peak I material is formed in the spin label reaction by the alkylation of the 3-nitrogen of the histidine residue at position 105.

The homogeneity of the peak I material was investigated by

chromatography on the analytical Bio-Rex 70 NaCl system of Crestfield (1963) (1.0 × 18 cm column). The 3-SLHis-105-RNase A was estimated to be better than 92% pure based on a ninhydrin analysis following alkaline hydrolysis of replicate aliquots taken from the effluent fractions (Fruchter and Crestfield, 1965). The 3-SLHis-105-RNase A elutes earlier than native RNase A which is to be expected if the substitution at histidine-105 leads to some loss in phosphate binding ability. When the peak I material (3-SLHis-105-RNase A) was assayed using yeast tRNA and 2′,3′-cyclic cyticylic acid as substrates, 86 ± 3% specific activity compared to native RNase A was obtained.

A highly purified sample of 3-SLHis-105-RNase A was obtained by pooling the appropriate tubes from a Bio-Rex 70 NaCl chromatography using a 1.0 × 25 cm column. The 3-SLHis-105-RNase A thus acquired and 3-SLHis-105-RNase A (peak I) which had not been further purified by Bio-Rex 70 NaCl chromatography both exhibited identical epr spectral line shapes, specific enzymatic activities, and amino acid analysis data. The results of these and additional experiments not reported on here indicated that the peak I material, as obtained from the spin-label reaction mixture separation chromatography, was sufficiently pure for use in the epr analysis of the conformational properties of the enzyme.

The 3-SLHis-105-RNase A was rapidly inactivated by iodoacetate at 40°, pH 5.5, under the conditions employed by Gundlach *et al.* (1959b). The rates of loss of activity for 3-SLHis-105-RNase A and native RNase A were in good agreement, demonstrating that the reactivities of the active-site histidines are not affected by the presence of the spin label at histidine-105. This experiment provides additional support for the assignment of His-105 as the site of attachment of the spin label.

The 3-SLHis-105-RNase A exhibited the epr spectrum shown in Figure 2b at pH 5.50. Comparison with the epr signal arising from the free nitroxide III in water (Figure 2a) demonstrates that the effective rotational correlation time of the spin label attached to histidine-105 of RNase A is much slower ($\tau_c \cong 1\text{–}5 \times 10^{-9}$ sec as opposed to $\tau_c = 4.9 \times 10^{-11}$ sec for Figure 2a).[2] In this intermediate tumbling region, it has been emphasized by Hamilton and McConnell (1968) that the spectra are exceedingly sensitive to the degree of motion of the radical.

Discussion

Smith (1968) has studied the reaction of the nitroxide III with ribonuclease A at pH 5.50 under reaction conditions identical to those utilized in the present study. Although Smith desalted the reaction mixture on Sephadex, the spin-labeled components were separated neither from one another nor from unreacted RNase A. Activity measurements on the desalted spin-label reaction mixture showed that 50% inactivation had occurred. Amino acid analysis of the performic acid oxidized mixture indicated loss of histidine and lysine with the appearance of 0.5 residue of 3-carboxymethylhistidine and 0.1 residue of ε-carboxymethyllysine. Based primarily on this evidence and on the fact that under similar reaction conditions iodoacetate alkylates the 3-nitrogen of histidine-12 leading to an inactive derivative (Crestfield *et al.*, 1963c,d), Smith concluded that the spin label III had likewise reacted at histidine-12 and that the resulting spin-labeled-histidine RNase A was totally inactive.

The results of our investigation show that in the reaction of the bromoacetamide nitroxide spin label III with bovine RNase A at pH 5.5, the chief products formed are in fact 3-SLHis-105-RNase A and a labile *S*-SLMet-RNase A, easily separable by chromatography on Bio-Rex 70. The 3-SLHis-105-RNase A derivative appeared slowly during the reaction at a rate comparable to that of the model reaction of iodoacetamide with free histidine (Fruchter and Crestfield, 1967). Preliminary amino acid analyses revealed that a single histidine was spin labeled at the imidazole 3-nitrogen in the principle product 3-SLHis-105-RNase A. On further characterization, a carboxyl-terminal peptide has been isolated which accounts for *all* of the spin label incorporated into the histidine-labeled enzyme. By the subtractive Edman degradative procedure, it has been demonstrated that the histidine at position 105 (amino terminal in the peptide) is the site at which the spin label is attached. Furthermore, this spin-labeled histidine derivative of RNase A is ~85% active compared to the native enzyme; alkylation of the spin-labeled enzyme with iodoacetate inactivates the substance at a rate comparable to the inactivation of native enzyme.

It is apparent from these data that the spin label does not exhibit the same specificity for histidine-12 as the related reaction of RNase A with iodoacetamide (Fruchter and Crestfield, 1967). Crestfield *et al.* (1963) have found that the reaction of iodoacetate with the "active-site" histidines of RNase A in the presence of polyvalent ions such as 0.2 M phosphate, 0.017 M sulfate, and cupric ion is either completely blocked or retarded to such an extent that slower alkylations on other less reactive amino acid side chains become competitive. It is unlikely that incomplete desalting in the preparation of RNase A for the labeling reaction could be the determining factor, since the RNase A used displayed the same reaction characteristics with iodoacetate as reported in the literature (Crestfield *et al.*, 1963c,d). Also, no deviation in the course of the reaction was observed when the spin labeling was carried out at pH 6.5 or in 0.01 M sodium acetate (pH 5.5), implying that acetate ion competition for histidines-12 and -119 has little or no significance in directing alkylation elsewhere. The reason for the lack of alkylation at either of the active-site histidines using the spin label III is not apparent although perhaps the course of the reaction is influenced by the steric and/or hydrophobic properties of the alkylating agent.

The pK_a's of the four histidines of RNase A have been determined and assigned by nuclear magnetic resonance studies (Meadows *et al.*, 1968; Roberts *et al.*, 1969). Histidine-105 ($pK_a = 6.7$) has the highest value of the four and from the point of view of its protonation state at pH 5.50 it should be the least reactive. Indeed, Bello and Nowoswiat (1971) have shown that bromoacetate alkylates RNase A at the 3-nitrogen of histidine-105 only if considerable time is allowed. Under the same conditions histidine-48, which is known to be buried, undergoes little or no reaction. As anticipated, the yield of the 3-SLHis-105-RNase A could be increased from 20 to 30% by carrying out the spin-labeling reaction at pH 6.5 where more of the histidine-105 exists as the nucleophilic species (in the unprotonated form).

When the 3-SLHis-105-RNase A reacted with iodoacetate

[2] The rotational correlation time, τ_c, for the free nitroxide III in water was calculated directly from the epr spectrum (Figure 2a) by the procedure of Stone *et al.* (1965) and represents the average of values obtained from the terms linear and quadratic in *M*. Limits for the rotational correlation time for the epr spectrum of 3-SLHis-105-RNase A (Figure 2b) were obtained by consideration of spectral line-shape computer simulations (Freed *et al.*, 1971; Goldman *et al.*, 1972; McCalley *et al.*, 1972).

at pH 5.5 under the conditions of Gundlach *et al.* (1959b) the enzymatic activity was lost due to alkylation of the active-site histidines. Furthermore, the 3-SLHis-105-RNase A behaved exactly as native RNase A when affinity chromatographed using 5′-(4-aminophenylphosphoryl)uridine 2′(3′)-phosphate coupled to Sepharose as the resin (Wilchek and Gorecki, 1969). It is consistent with these observations to conclude that the conformational integrity of the active site is still preserved in spite of the presence of the spin label attached to histidine-105. The paramagnetic probe at histidine-105 has not drastically perturbed the biological macromolecule. However, the lowered specific activity of the 3-SLHis-105-RNase A suggests that a definite interaction exists between the attached spin label and the active site of the molecule—an interaction which presumably should enable the spin label to sense conformational changes which occur in the active center. The apparent rotational correlation time, τ_c, of the epr spectrum of the 3-SLHis-105-RNase A (Figure 2b) is shorter than that for the *S*-SLMet-RNase A epr spectrum (Figure 2c). Thus, the spin label at histidine-105 is not totally immobilized relative to the protein; rather, it must have some residual motion with which to sense its surroundings. The spin label's ability to yield information about the conformational properties of the active center of RNase A will be considered in a subsequent paper which will discuss the effects on the epr spectrum of 3-SLHis-105-RNase A brought about by a variety of physical and chemical modifications of the labeled enzyme.

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References

Barnard, E. A. (1969), *Annu. Rev. Biochem. 38*, 677.

Bello, J., and Nowoswiat, E. F. (1971), *Eur. J. Biochem. 22*, 225.

Crestfield, A. M. (1963), *Anal. Chem. 35*, 1762.

Crestfield, A. M., Moore, S., and Stein, W. H. (1963a), *J. Biol. Chem. 238*, 622.

Crestfield, A. M., Stein, W. H., and Moore, S. (1963b), *J. Biol. Chem. 238*, 618.

Crestfield, A. M., Stein, W. H., and Moore, S. (1963c), *J. Biol. Chem. 238*, 2413.

Crestfield, A. M., Stein, W. H., and Moore, S. (1963d), *J. Biol. Chem. 238*, 2421.

Crook, E. M., Mathias, A. P., and Rabin, B. R. (1960), *J. Biochem (Tokyo) 74*, 234.

Freed, J. H., Bruno, G. V., and Polnaszek, C. F. (1971), *J. Phys. Chem. 75*, 3385.

Fruchter, R. G., and Crestfield, A. M. (1965), *J. Biol. Chem. 240*, 3868.

Fruchter, R. G., and Crestfield, A. M. (1967), *J. Biol. Chem. 242*, 5807.

Goldman, S. A., Bruno, G. V., Polnaszek, C. F., and Freed, J. H. (1972), *J. Chem. Phys. 56*, 716.

Griffith, O. H., and Waggoner, A. S. (1969), *Accounts Chem. Res. 2*, 17.

Gross, E., and Witkop, B. (1962), *J. Biol. Chem. 237*, 1856.

Gundlach, H. G., Moore, S., and Stein, W. H. (1959a), *J. Biol. Chem. 234*, 1761.

Gundlach, H. G., Stein, W. H., and Moore, S. (1959b), *J. Biol. Chem. 234*, 1754.

Hamilton, C. L., and McConnell, H. M. (1968), *in* Structural Chemistry and Molecular Biology, Rich, A., and Davidson, N., Ed., San Francisco, Calif., W. H. Freeman, p 115.

Hermans, J., Jr., and Scheraga, H. A. (1961), *J. Amer. Chem. Soc. 83*, 3283.

Hirs, C. H. W., Moore, S., and Stein, W. H. (1956), *J. Biol. Chem. 219*, 623.

Kartha, G., Bello, J., and Harker, D. (1967), *Nature (London) 213*, 862.

Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237*, 2547.

Kunitz, M. (1946), *J. Biol. Chem. 164*, 563.

McCalley, R. C., Shimshick, E. J., and McConnell, H. M. (1972), *Chem. Phys. Lett. 13*, 115.

McConnell, H. M., and McFarland, B. G. (1970), *Quart. Rev. Biophys. 3*, 91.

Meadows, D. H., Jardetzky, O., Epand, R. M., Ruterjans, H. H., and Scheraga, H. A. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 766.

Morrisett, J. D. (1968), Ph.D. Thesis, University of North Carolina, Chapel Hill, N. C.

Roberts, G. C. K., Meadows, D. H., and Jardetzky, O. (1969), *Biochemistry 8*, 2053.

Rozantzev, E. G., and Krinitzkaya, L. A. (1965), *Tetrahedron 21*, 491.

Smith, I. C. P. (1968), *Biochemistry 7*, 745.

Smyth, D. G., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238*, 227.

Stone, T. J., Buckman, T., Nordio, P. L., and McConnell, H. M. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 1010.

Wilchek, M., and Gorecki, M. (1969), *Eur. J. Biochem. 11*, 491.