A Study of the Conformational Properties of Bovine Pancreatic Ribonuclease A by Electron Paramagnetic Resonance*

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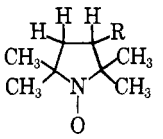
ABSTRACT: A technique is reported for spin labeling bovine pancreatic ribonuclease A and using electron paramagnetic resonance spectroscopy to study its conformational properties. Bromo acid nitroxide and bromo amide nitroxide spin labels react with the histidine residues at the active site of the enzyme, and to a much lesser extent with lysine residues on the exterior of the protein molecule. A five-membered maleimide nitroxide spin label reacts only with these lysine residues, whereas a six-membered maleimide nitroxide label reacts with lysine-41 at the active site. Transition temperature measurements indicate that the spin-labeled derivatives are structurally very similar to native ribonuclease (RNase) A.

Evidence for the binding of substrate ribonucleic acid (RNA) and synthetic polyribonucleotides to spin-labeled RNase A, RNase S, and RNase S protein is presented; these data indicate that although enzymic action has been suppressed by labeling, the sites involved in recognition and binding of substrates are still intact and operational. Direct observation of the response of spin labels in the region of the active site when various perturbations are applied to RNase yields results in accord with current views of the molecular structure. However, perturbations of the tyrosine residues do not always correlate with perturbations of the active site. RNase S appears to have an active site environment very similar to that of RNase A. The temperature dependence of the electron paramagnetic resonance spectra yields activation energies related to the expansion of the active site; the magnitudes of these energies indicate that only a small number of bonds must be disrupted to destroy the three-dimensional structure necessary for enzymic activity.

Recently a technique has been developed for attaching small paramagnetic species, spin labels, to large molecules of biological significance (Stone *et al.*, 1965; Griffith and McConnell, 1965; Stryer and Griffith, 1965; Berliner and McConnell, 1966; Boeyens and McConnell, 1966; Ohnishi *et al.*, 1966), and using the resulting electron paramagnetic resonance spectrum to study the properties of the biological system. The spin labels have been of the general structure



where R is a particular functional group. The electron paramagnetic resonance spectrum of the unattached label has a simple hyperfine pattern of three lines of equal intensity due to interaction of the unpaired electron with the ^{14}N nucleus (Figure 1). However, due to considerable anisotropy in the g and hyperfine tensors (Griffith *et al.*, 1965), the electron paramagnetic resonance spectra are very sensitive to the rate and nature of the rotational motion of the spin labels. Briefly, the more restricted the motion of the spin label, the more asymmetric and the broader will be the electron paramagnetic resonance hyperfine lines. A further advantage of this technique is that very low concentrations of paramagnetic species can be detected (down to approximately 10^{-7} M), so that even if spin labeling is not complete, only the properties of the spin-labeled species are studied. In this communication we report the application of the spin-labeling method to bovine pancreatic ribonuclease A.

A very considerable amount of information on the three-dimensional structure of RNase A, and the relationship between this structure and the catalytic function of the enzyme, has already been obtained by chemical and spectroscopic methods (Scheraga and Rupley, 1962; Stein, 1964). In particular, three amino acid residues have been implicated in the active site of the enzyme (histidine-12, histidine-119, and lysine-41) (Crestfield *et al.*, 1963a,b; Heinrickson *et al.*, 1965; Heinrickson, 1966; Hirs *et al.*, 1961). The activity of RNase is thought to be due to the proximity of these three residues; perturbations which cause a

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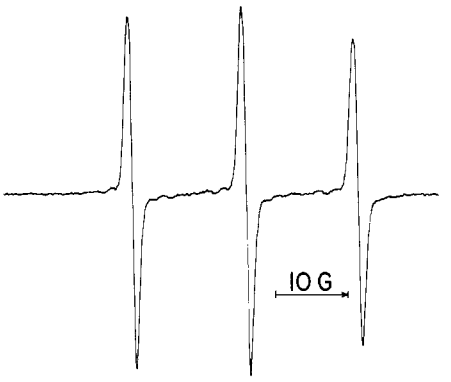


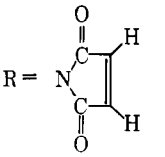
FIGURE 1: Electron paramagnetic resonance spectrum of the bromo acid spin label I in 0.10 M acetate buffer (pH 5.5).

disruption of the active site cause a concomitant loss of enzymic activity. The aim of the present study was therefore to develop a spin label for the active site of RNase and to use its electron paramagnetic resonance spectrum as an indicator of the integrity of the active site region when RNase is exposed to the various modifications known to effect its activity and physicochemical properties.

RNase has been shown to react with bromoacetic acid in acetate buffer at pH 5.5 to form inactive derivatives which are carboxymethylated either at the one position of histidine-119 or the three position of histidine-12 (Crestfield *et al.*, 1963a,b). The yield of the histidine-119 derivative was about eight times that of the histidine-12 derivative. Carboxymethylation of one histidine prevented reaction with the other. It was also demonstrated that the higher homologs of bromoacetic acid reacted with the histidine residues at the active site of RNase, but at rates decreasing with increasing molecular size. Stark *et al.* (1961) showed that no structural change detectable by spectrophotometric titration had occurred on carboxymethylation. Heinrickson (1966) has also reported that if the reaction of RNase with bromoacetate is carried out at pH 8.5 carboxymethylation of lysine-41 is the dominant reaction, with carboxymethylation of histidines-119 and -12 occurring to a lesser extent. The relative reactivities of the histidine and lysine residues at pH 8.5 varied with the size of the higher homologs. If the reaction at pH 8.5 was done with iodoacetamide, however, only derivatives carboxamidomethylated at lysine resulted; mono- and dicarboxamidomethyllysine-41 were the major products, with the lysine-1 derivatives being formed to a lesser extent.

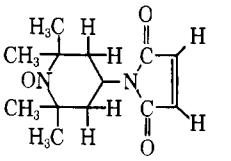
As analogs of the bromo acids the following nitroxide spin labels were prepared: R = $NHC(=O)CH_2CH(Br)COOH$ and R = $NHC(=O)CH(Br)CH_2COOH$ (I). The bromo amide nitroxide, R = $NHC(=O)CH_2Br$ (II), as prepared by Ogawa and McConnell (1967), was used as an analog of iodoacetamide.

N-Ethylmaleimide has been used successfully for labeling sulfhydryl groups (Benesch and Benesch, 1961) and amino groups (Sharpless and Flavin, 1966). Recent work by McConnell and coworkers has indicated that the maleimide nitroxide spin label (structure III) reacts with sulfhydryl and ϵ-amino groups. As native



III

RNase A contains no sulfhydryl groups, label III was chosen to compare the results of spin labeling only lysine residues with those obtained using spin labels I and II. Compound III suffers from the disadvantage of rapid hydrolysis, so the six-membered maleimide nitroxide IV (Ohnishi *et al.*, 1966) was used in some experiments.



IV

Experimental Procedure

Synthesis of the Spin Labels. The bromo acid spin labels I were prepared by reaction of bromosuccinic anhydride with 2,2,5,5-tetramethyl-3-aminopyrrolidone-1-oxyl (Stone *et al.*, 1965). Attempts to prove that the product was a mixture of α- and β-bromo acids were unsuccessful, and the labels I were used as obtained from the reaction mixture by crystallization from ethyl ether. Labels II and III were prepared according to the methods of Ogawa and McConnell (1967) and Griffith and McConnell (1965), respectively. Compound IV was obtained from Dr. W. C. Landgraf of Varian Associates.

Materials. Bovine pancreatic ribonuclease A (Code RAF, phosphate free) was obtained from the Worthington Biochemical Corp. and used without further purification. Mann Laboratories supplied oxidized RNase and 1-carboxymethylhistidine-119 ribonuclease (prepared according to the method of Crestfield *et al.* (1963a) by alkylation with iodoacetate). The RNA used was the highly polymerized preparation from yeast supplied by Calbiochem and Worthington. Synthetic polyribonucleotides were obtained from Miles Laboratories, Inc. RNase S and RNase S protein were generously supplied by Professor F. M. Richards, Yale University.

Spin-Labeling Techniques. In a typical spin-labeling experiment 2–10 mg of the appropriate spin label was added to 15 mg of RNase A in 1 ml of the desired

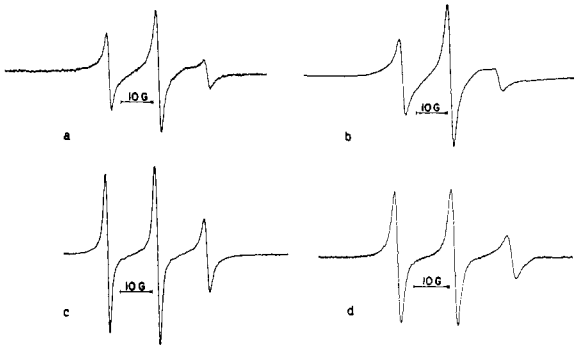


FIGURE 2: Electron paramagnetic resonance spectra of RNase spin labeled in acetate buffer, pH 5.5, with: (a) bromo acid spin label I, (b) bromo amide spin label II, (c) five-membered maleimide spin label III, and (d) six-membered maleimide spin label IV.

buffer. To eliminate dimers the solution of RNase was held at 65° for 1 hr, then cooled slowly to room temperature before addition of the spin label. The solution was stirred at room temperature in a light-free container for from 1 to 10 days. The bromo acid label I reacted slowly with RNase, requiring 10 days to obtain a workable electron paramagnetic resonance intensity. Reaction with the bromo amide label II was faster, and required only several days. For the maleimide spin label III only several hours of reaction were possible, since the compound hydrolyzed completely in water in this time; this difficulty did not occur with IV, and reaction times of several days were used. After the desired reaction time the excess spin label was removed by gel filtration on Sephadex G-25, or by exhaustive dialysis.

Assays, Amino Acid Analyses, and Instrumentation. Activity assays were carried out by measuring the increase in optical density at 284 mμ when cytidine 2′,3′-cyclic phosphate is hydrolyzed to cytidine 3′-phosphate (Crook *et al.*, 1960). The methods of Kunitz (1946) and Kalnitsky *et al.* (1959) were also used. The first method gave the most reproducible results and was used routinely. Amino acid analyses were carried out by Dr. Leon M. Krausz of Rutgers University on a Beckman Model 120B using the method of Moore *et al.* (1958). Before analysis the samples of spin-labeled RNase were oxidized with performic acid at 0° according to Hirs (1956), and then hydrolyzed in 6 M HCl at 110° for 22 hr. The major instruments used in this study were a Varian V-4500 electron paramagnetic resonance spectrometer operating at 9.5 GHz with 100-kHz modulation, a Cary 15 spectrophotometer, and a Radiometer 22 pH meter.

Experimental Results

Reaction of RNase A with I, II, III, and IV. The electron paramagnetic resonance spectra shown in Figure 2 are due to RNase A spin labeled with I, II, III, or IV in 0.1 M acetate buffer at pH 5.5.[1] All four spectra have the basic three-line hyperfine pattern, but the three lines have different peak height ratios and the spectra contain differing amounts of the broad components between the principal lines. Blocking experiments, described later, indicate that the spectra 2a and 2b consist of a superposition of two types of spectra due to labeling at least two different sites in the protein molecule. The broader of the two spectra will be assigned to spin labels at the active site; corroborative evidence is presented in later sections. Spectrum 2c, due to RNase-III, does not contain any broad components, and is due to spin labeling lysine residues situated on the exterior of the protein molecule. Spectrum 2d, due to reaction with the maleimide label IV, does contain broad components; they are thought to arise from labeling a lysine residue in a restricted environment, tentatively assigned to be lysine-41. Estimated degrees of labeling, measured from electron paramagnetic resonance intensities, are: RNase-I, 50%; RNase-II, 50%; RNase-III, 75%; and RNase-IV, 35%.

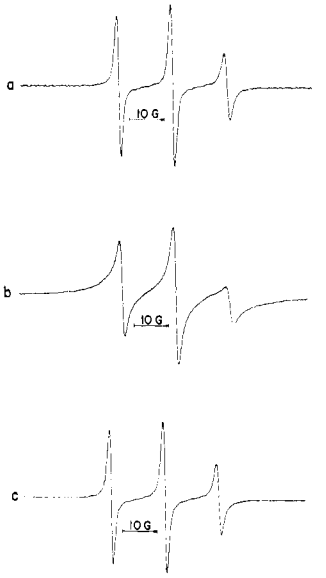


FIGURE 3: Electron paramagnetic resonance spectra of RNase spin labeled in 0.02 M Veronal buffer, pH 8.5, with: (a) bromo acid spin label I, (b) bromo amide spin label II, and (c) maleimide spin label III. All spectra were recorded after changing the solvent to 0.10 M acetate buffer, pH 5.5.

When native RNase is treated with I, II, and III in 0.02 M Veronal buffer (pH 8.5), the resulting electron paramagnetic resonance spectra (Figure 3) are considerably different from those obtained by reaction at pH 5.5 (after reaction at pH 8.5 the excess spin

[1] Henceforth we shall refer to the derivatives obtained by reaction of RNase A with the various spin labels as RNase-I, RNase-II, RNase-III, and RNase-IV, where the roman numeral corresponds to the spin labels defined above.

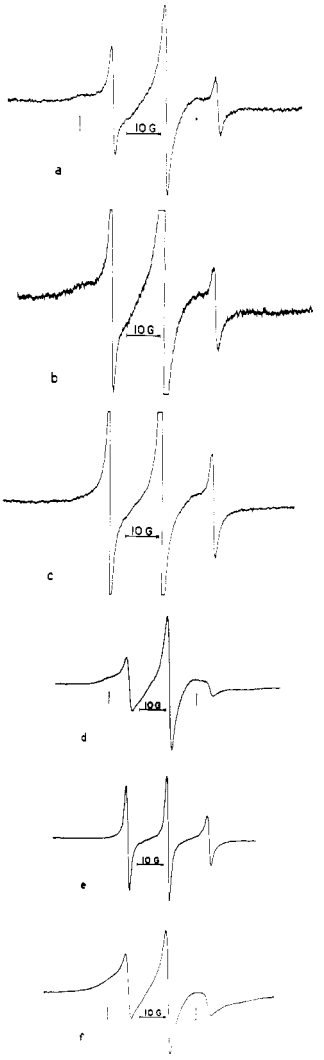


FIGURE 4: Various effects of RNA on the electron paramagnetic resonance spectrum of RNase. (a) Effect of RNA addition on the electron paramagnetic resonance spectrum of RNase spin labeled at pH 5.5 with the bromo acid spin label I; (b) same solution as a, but under higher instrument gain conditions; (c) electron paramagnetic resonance spectrum of solution used for a, before RNA addition, under high instrument gain conditions; (d) effect of RNA addition on the electron paramagnetic resonance spectrum of RNase spin labeled at pH 5.5 with the bromo amide spin label II (compare with Figure 2b); (e) effect of RNA on the electron paramagnetic resonance spectrum of RNase spin labeled at pH 5.5 with the maleimide spin label III (compare with Figure 2c); and (f) effect of RNA addition on the electron paramagnetic resonance spectrum of RNase spin labeled at pH 8.5 with the bromo amide spin label II (compare with Figure 3b).

label was removed by gel filtration on a Sephadex G-25 column equilibrated with acetate buffer, pH 5.5). In the case of reaction with I and II, the electron paramagnetic resonance spectra 3a and 3b have narrower lines, the peak height ratios are nearer the values expected for no broadening due to restricted motion, and the broad components between the three principal lines are less pronounced than in the case of reaction at pH 5.5. These observations indicate that both spin labels I and II have greater mobility if treated with RNase at pH 8.5. The effect is more drastic in the spectra due to RNase-I. Reaction of RNase with III, the maleimide spin label, at pH 8.5 results in an electron paramagnetic resonance spectrum more intense than that obtained at pH 5.5, but similar in shape (Figure 3c).

Interaction of Spin-Labeled RNase with RNA and Polynucleotides. If RNA is added to the samples responsible for the electron paramagnetic resonance spectra of Figure 2, spin labeled at pH 5.5, the spectra of Figure 4 result. In the case of RNase-I and RNase-II distinct spectral changes, due to decreased mobility of the spin label, are obvious (the changes are indicated by vertical arrows in Figure 4a,d). With the maleimide spin label III the electron paramagnetic resonance spectra before and after RNA addition are exactly superimposable; with label IV, addition of RNA causes a broadening of all lines and increased asymmetry of the electron paramagnetic resonance spectrum, but to a lesser extent than with RNase-I and RNase-II.

Addition of RNA to RNase spin labeled at pH 8.5 produces different results. With the bromo acid label I there is no noticeable change in the electron paramagnetic resonance spectrum. In the case of the bromo amide label II there is a marked effect (Figure 4f) similar in character to that observed after spin labeling at pH 5.5, but with the broad lines (indicated with vertical arrows in Figure 4f) appearing at a slightly higher field value than in the pH 5.5 spectra. Addition of RNA to solutions of RNase labeled with III at pH 8.5 produced no detectable change in the electron paramagnetic resonance spectra.

In all cases where addition of RNA produced a detectable change in the electron paramagnetic resonance spectrum of spin-labeled RNase, the effect persisted even after weeks of storage at 0°. It could *not* be removed by addition of native RNase.

Addition of cytidylic acid (Sigma Chemicals, 2′ and 3′ mixed isomers) or a solution of RNA core (oligonucleotides produced by a 24-hr digestion of RNA by RNase) to solutions of RNase-I, -II, or -III at pH 5.5 produced no discernible change in the electron paramagnetic resonance spectra.

Small quantities of polyadenylic acid (poly A) and polycytidylic acid (poly C) produced changes in the electron paramagnetic resonance spectrum of RNase-II similar to those caused by RNA addition. Large amounts of polyuridylic acid (poly U) also resulted in the appearance of broad components in the electron paramagnetic resonance spectrum of RNase-II, but this was due primarily to an increase in solution viscosity and could be removed by threefold dilution of the reaction mixture. Polyguanylic acid (poly G) produced the largest effect on the electron paramagnetic resonance spectrum of RNase II, the result resembling

that shown in Figure 6b but with the broad low-field component better resolved from the first principal line. Almost as strong an effect was produced by addition of polyinosinic acid (poly I) to RNase-II. In all the above experiments, whenever a positive effect due to addition of polynucleotides was observed, it was not considered intrinsic unless it was insensitive to a ten-fold dilution of the solution. All spectral changes induced by addition of synthetic polyribonucleotides could be reversed by addition of active RNase A, or by allowing the residual RNase activity to operate overnight. The spectral changes could then be restored immediately by addition of more polynucleotide. RNase-I behaved in a similar manner to RNase-II upon addition of polynucleotides. Addition of poly A or poly G to solutions of RNase-IV produced electron paramagnetic resonance spectral changes similar to those caused by substrate RNA, that due to poly G being the greater effect.

Reaction of 1-Carboxymethylhistidine-119-ribonuclease (1-CMHis-119-RNase) with I, II, and III. The electron paramagnetic resonance spectra obtained when 1-CMHis-119-RNase is treated with I, II, and III at pH 5.5 are shown in Figure 5. Similar, but considerably more intense, spectra were obtained when the reactions were performed at pH 8.5 in 0.02 M Veronal buffer. The spectrum due to reaction with the bromo acid label I is of considerably weaker intensity than that obtained with native ribonuclease, the peak height ratios of the three principal lines correspond to a more mobile spin label, and there is relatively less of the broad components. Exactly the same observations may be made in comparing the spectra due to reaction of the bromo amide label II with native ribonuclease and its carboxymethylated derivative. A comparison of the results of reaction with maleimide spin label III shows that the spectra are essentially identical (the spin label has a similar degree of mobility in both cases).

On addition of RNA to solutions of 1-CMHis-119-RNase spin labeled with I, II, or III, in acetate buffer at pH 5.5 or 8.5, the electron paramagnetic resonance spectra underwent no detectable changes.

Reaction of RNase S and S Protein with II. Samples of RNase S and S protein were treated with II at pH 5.5 in 0.1 M acetate buffer. The electron paramagnetic resonance spectrum of RNase S-II is similar to that shown in Figure 2a (compare with Figure 2b, due to RNase-II under similar conditions). Addition of RNA caused a shift in the broad spectral components similar to that observed with RNase-I and -II. The electron paramagnetic resonance spectrum of RNase S protein-II was very similar to that obtained from RNase-IV (Figure 2d), *i.e.*, not nearly as much broad spectral component as with RNase S-II. Addition of RNA caused a slight broadening of all electron paramagnetic resonance lines in a manner similar to that observed with RNase-IV.

Spin-Labeled Oxidized RNase. Oxidized RNase labeled with II at pH 5.5 resulted in an electron paramagnetic resonance spectrum due to a mobile spin label (similar to Figure 2c) which was insensitive to addition of RNA. Similar results were obtained with a sample of RNase labeled with II and subsequently oxidized with performic acid.

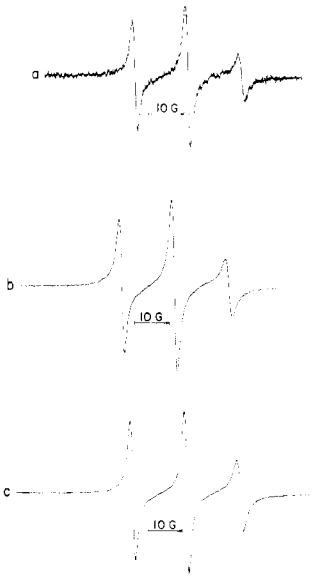


FIGURE 5: Electron paramagnetic resonance spectra of 1-carboxymethylhistidine-119-ribonuclease spin labeled at pH 5.5 in acetate buffer with: (a) bromo acid spin label I, (b) bromo amide spin label II, and (c) maleimide spin label III.

Influence of pH on the Electron Paramagnetic Resonance Spectra of RNase Spin Labeled with I and II at pH 5.5. The effects of varying the pH of solutions of RNase-I and -II were similar, and therefore only the details and spectra for RNase-II are presented. On lowering the pH by increments of 0.5 pH unit, the lines in the electron paramagnetic resonance spectrum due to RNase-II gradually became narrower, and the relative intensity of the broad components decreased (Figure 6a). Narrowing of the spectral lines was most noticeable at and below pH 3.0. Raising the pH by increments of 0.5 resulted in broadening of the principal electron paramagnetic resonance lines and reappearance of broad components. Surprisingly, however, when the pH was raised to 3.5 a broad component appeared to low field of the lowest field principal line (marked by an arrow in Figure 6b). Further increases in pH resulted in intensification of all broad components at the expense of the narrow components, until at pH 5.5 the electron paramagnetic resonance spectrum was as shown in Figure 6b (compare with Figure 2b, due to RNase-II at pH 5.5 before pH variation). That is, the spin label had considerably less mobility after the pH cycle from 5.5 to 2.0 to 5.5. The broad components could be removed by lowering the pH to 2.5 again, but on raising it to 5.5 the electron paramagnetic resonance spectrum always had the appearance of Figure 6b. Starting from pH 5.5 and proceeding gradually to 8.5 had no effect on the electron para-

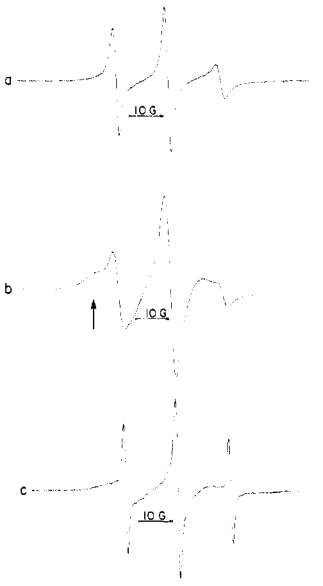


FIGURE 6: Influence of pH change on the electron paramagnetic resonance spectrum of RNase labeled with the bromo amide spin label II at pH 5.5 (compare with Figure 2b). (a) pH 2.5, (b) pH 5.5 after pH 2.5, and (c) pH 11.

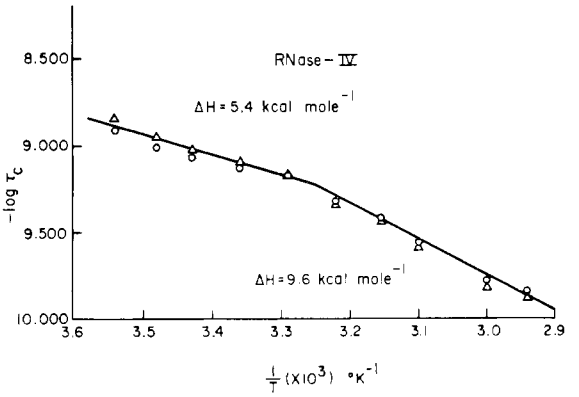


FIGURE 7: Dependence of the spin label correlation time upon absolute temperature for RNase-IV. The circles and triangles represent correlation times calculated from the linear and quadratic terms in the nitrogen spin quantum member, respectively (see text).

magnetic resonance spectrum. Raising the pH further up to 11 (irreversible denaturation) resulted in the immediate appearance of spectrum 6c. This spectrum corresponds to much greater mobility of the spin label than spectrum 6a or 6b. Lowering the pH to 5.5 resulted in no change in the electron paramagnetic resonance spectrum if the system had remained at pH 11 for an appreciable length of time (more than several minutes).

On lowering the pH of solutions of spin-labeled 1-CMHis-119-RNase from 5.5 to 2.5, then raising the pH back to 5.5, no appreciable change in the electron paramagnetic resonance spectrum was noticeable. Raising the pH to 11 resulted in an irreversible narrowing of all spectral lines.

Influence of Urea on the Electron Paramagnetic Resonance Spectra of RNase-I, -II, and -III. Addition of urea in increments such as to make its concentration 4 M, 8 M, and saturated had surprisingly little effect on the electron paramagnetic resonance spectrum of RNase labeled with the bromo acid spin label I in acetate buffer at pH 5.5. The only detectable effect was a slight broadening of all spectral lines. Similar treatment of solutions of RNase labeled with the bromo amide spin label II resulted in a narrowing of all spectral lines, and culminated in the disappearance of the broad components in saturated urea. At 4 M urea the spectra were still unchanged but the lines narrowed considerably in going from 4 to 8 M. With both RNase-I and -II, removal of urea by gel filtration on Sephadex G-25 resulted in spectra identical with those before urea addition. Urea addition had no detectable effect on the electron paramagnetic resonance spectrum of RNase-III. If a solution of RNase-II was first treated with sufficient RNA to effect an electron paramagnetic resonance spectral change, and then made 8 M in urea, the electron paramagnetic resonance spectral lines narrowed to almost the same extent as when RNA was absent. Removal of the urea by dialysis resulted in the original electron paramagnetic resonance spectrum, and not that occurring after RNA addition. Upon addition of a second portion of RNA the electron paramagnetic resonance spectrum demonstrated the usual RNA effect. When a solution of RNase-II was first made 8 M in urea, and then RNA added, the electron paramagnetic resonance spectral lines were only slightly broader than in 8 M urea without RNA.

Addition of urea to solutions of 1-CMHis-119-RNase, spin labeled with II at pH 8.5, caused no change in the electron paramagnetic resonance spectrum (in marked contrast to RNase-II under the same conditions).

Reaction of RNase with I in 8 M Urea and in Phosphate Buffer. When the bromo acid spin label I is treated with RNase at pH 5.5 in acetate buffer which is 8 M in urea the resulting electron paramagnetic resonance spectrum is much stronger than usual and has a form similar to that of Figure 3a, the principal lines are relatively narrow, and there are no broad components between them. Addition of RNA to this solution produced no change in the electron paramagnetic resonance spectrum, either in the presence or absence of urea.

If RNase is treated with I in 0.05 M phosphate buffer (pH 5.5), the resulting electron paramagnetic resonance spectrum is much weaker than in the case of reaction in acetate buffer at pH 5.5. The three principal lines are narrower than in the latter case, there is no detectable broad component in the spectrum, and addition of RNA to the solution causes no change in the electron paramagnetic resonance spectrum. If, however, RNase is treated with I in acetate buffer and the buffer changed

TABLE I: Amino Acid Analysis Results.[a]

	Theoretical	RNase A	RNase-I	RNase-II pH 5.5	RNase-II pH 8.5	RNase-III
Lysine	10	9.9	10.1	8.9	9.3	10.0
Histidine	4	3.7	4.3	2.9	3.4	3.8
Methionine sulfone	4	3.8	3.8	3.7	4.0	4.1
3-CM-histidine	0	0	0	0.5	0.2	0
1-CM-histidine	0	0	0	0	0	0
ε-CM-lysine[b]	0	0	0	0.1	0.6	0
ε-DiCM-lysine[b]	0	0	0	0	0.3	0

[a] Short-column results based on arginine, long column on aspartic acid. [b] Calibration constant for glycine used.

to 0.05 M phosphate by gel filtration on Sephadex G-25, the electron paramagnetic resonance spectrum contains the usual amount of broad component, and addition of RNA results in the appearance of the broad line at low field.

Effect of Temperature on the Optical and Electron Paramagnetic Resonance Spectra of Spin-Labeled Derivatives. The optical spectra of RNase A and its derivatives with spin labels I, II, III, and IV were compared. The spectral shapes, and extinction coefficients at 2775 Å, were not detectably different.

The temperature dependence of the optical density at 2870 Å of RNase A, RNase-I, and RNase-II was determined by Dr. T. Schleich, Dartmouth College, using a temperature-programmed Gilford 4000 spectrophotometer. The solutions were made up in 0.1 M NaCl and 0.01 M sodium cacodylate buffer (pH 6.8). The samples of RNase-I and RNase-II were similar to those used in the electron paramagnetic resonance studies, and therefore contained approximately 50% of the labeled derivative and 50% of intact RNase A. The measured transition temperatures were: RNase A, 62.9°; RNase-I, 61.7°; and RNase-II, 59.8°.

Electron paramagnetic resonance spectra of RNase-II, -III, and -IV were measured over the temperature range 5–85°. With RNase-II and -IV the electron paramagnetic resonance spectral lines became considerably narrower as the temperature was increased; in the case of RNase-II the broad spectral components (Figure 2b) had disappeared entirely by 40°. With RNase-III, the derivative with the five-membered maleimide nitroxide label, only a very slight narrowing of the electron paramagnetic resonance lines was observed. The temperature response in all three cases was reversible if the solutions were not held at elevated temperatures (>65°) for prolonged periods (>0.5 hr). Correlation times for the rotational motion of the spin labels were calculated using the formalism of Kivelson (1960) as presented by Stone *et al.* (1965). Separate calculations of the correlation time (τ_c) were made using the terms linear and quadratic in the nitrogen spin quantum number. Care was taken to use very low microwave power while recording the spectra since saturation effects can lead to erroneous correlation times derived from the term linear in the nitrogen spin quantum number (B. Hoffman, personal communication). Correlation times calculated by the two methods differed only slightly (see Figure 7).

In Figure 7 we present the relationship between log τ_c and the reciprocal absolute temperature for RNase-IV (the six-membered maleimide spin label derivative); a similar curve was obtained for RNase-II (the bromo amide spin label derivative). It is apparent that two separate activation energies are manifest: at low temperatures 5.4 kcal/mole, at higher temperatures 9.6 kcal/mole. The curves for RNase-II yielded activation energies of 5.5 and 10.8 kcal/mole. In both cases the crossover point was 35°. A similar plot for RNase-III gave a single line of very gentle slope and an activation energy of 0.33 kcal/mole.

Activity Measurements and Amino Acid Analyses. Activity measurements showed that both RNase-I and RNase-II had 50% of the specific activity due to the control RNase samples; RNase-III had only 20%, and RNase-IV had 40%. A control sample of 1-CM-His-119-RNase had no detectable enzymic activity.

The hydrolysis procedure used to prepare labeled RNase samples for analysis is expected to result in cleavage of the amide or imide bonds connecting the nitroxide and attaching moieties of the spin labels. Thus we expect to find modified amino acid residues appearing on the chromatogram for all the derivatives; with I, III, and IV, these would be carboxyacetoxymethyl derivatives, whereas with II they would be the usual carboxymethyl derivatives (Crestfield *et al.*, 1963a,b; Heinrickson *et al.*, 1965). The results for the amino acids of interest are presented in Table I.

We see from Table I that in the case of the product with I the lysine and histidine assays come very close to the values for unmodified RNase A. No other normal amino acids were unusually low, no unexpected peaks were found, and no peaks were detectable at the elution volumes of the dicarboxyalkyl derivatives of histidine and lysine. However, activity measurements indicate that reaction of RNase with I results in a 50% loss of activity, comparable with that caused by

II. Thus, it appears that the modified amino acids resulting from labeling with I are hydrolyzed back to the normal amino acids during sample preparation. In the case of RNase labeled with II, however, the analyses indicate loss of histidine and lysine and the presence of detectable amounts of 3-carboxymethylhistidine and ε-carboxymethyllysine. The analyses of RNase-III indicate that the modified amino acids did not withstand acid hydrolysis. Intensive efforts to detect weak peaks over the entire elution volume range were made.

Labeling of RNase-III with II. A sample of RNase-III was labeled with the bromo amide label II at pH 5.5. The resulting electron paramagnetic resonance spectrum was very similar to that ordinarily obtained when native RNase A is treated with II (Figure 2b), which is drastically different from that of RNase-III before labeling with II (Figure 2c). Addition of RNA had no effect upon the electron paramagnetic resonance spectrum.

Discussion

RNase Spin Labeled with the Bromo Acid Nitroxide I. In view of the results of Heinrickson *et al.* (1965) on the influence of increasing molecular size on the reactivity of the α-bromo acids toward the active site of RNase it is evident that the spin labels I should be much less reactive than bromoacetic acid. This is borne out by the long time required for reaction with I. One would not expect the reaction to be entirely specific for the histidine residues at the active site, and might expect other reactions to occur upon prolonged exposure. The appearance of two components in the spectrum due to RNase spin labeled with I at pH 5.5 (Figure 2a), only one of which is affected by addition of RNA (Figure 4a), and the fact that I reacts with 1-CMHis-119-RNase to yield a low-intensity electron paramagnetic resonance spectrum of narrower lines (Figure 5a), indicate that some site other than histidine-119 or -12 has been spin labeled by I. This site is presumably a lysine residue, but the absence of an RNA effect on the electron paramagnetic resonance spectra due to native RNase or 1-CMHis-119-RNase labeled with I at pH 8.5 argues against lysine-41. It must be concluded therefore that at both pH 5.5 and 8.5 spin label I reacts with one or more lysine residues on the exterior of the protein molecule. The observation that reaction of 1-CMHis-119-RNase with I at pH 8.5 produces electron paramagnetic resonance spectra similar in form to, but more intense than, those obtained by reaction at pH 5.5 supports this conclusion. Further corroboration is rendered by the similarity between the electron paramagnetic resonance spectra due to 1-CMHis-119-RNase labeled with I at pH 5.5 and 8.5 and those due to RNase and 1-CMHis-119-RNase treated with the five-membered nitroxide maleimide spin label III at pH 5.5 and 8.5 (compare Figures 3a and 5a with Figures 2c, 3c, and 5c). The positive effect of RNA on the broad component of the electron paramagnetic resonance spectrum, and the absence of this component and an RNA effect in the spectrum due to reaction of I with 1-CMHis-119-RNase, combined with the chemical evidence for reaction of α-bromo acids with histidines-119 and -12 support assignment of the broader electron paramagnetic resonance spectral component to a spin label attached to either histidine-119 or -12. The similarity between the results for labels I and II, and the positive evidence that II reacts with His-12 of RNase A, suggests His-12 as the most likely site of reaction, but this remains to be confirmed by chromatography.

It was found that phosphate ion inhibited alkylation of the histidine residues of ribonuclease by iodoacetate (Crestfield *et al.*, 1963b). A similar inhibition was found for alkylation at pH 5.5 by D-α-bromo-*n*-butyrate (Heinrickson *et al.*, 1965). Spin labeling of RNase by I in 0.05 M phosphate buffer yielded a weak electron paramagnetic resonance spectrum of narrow lines with no broad component, which was insensitive to RNA addition. This is further evidence in support of assigning the broad component in the electron paramagnetic resonance spectrum of RNase-I, labeled in acetate buffer at pH 5.5, to a spin-labeled histidine residue at the active site.

Heinrickson (1966) showed that the reactivities of α-bromo acids toward the histidine residues at the active site of ribonuclease were generally lower when the reaction was carried out at pH 8.5. This, combined with the decreased reactivity of I relative to the compounds studied by Heinrickson, accounts for the absence of a broad component in the electron paramagnetic resonance spectrum of RNase labeled with I at pH 8.5 and for the absence of a positive RNA effect on the spectrum. Apparently I does not react with lysine-41 at this pH, but does react weakly with other lysine residues on the exterior of the protein structure (Figure 3a).

Barnard and Stein (1960) reported that RNase is inactivated rapidly by bromoacetic acid in 8 M urea at pH 5.5. Stark *et al.* (1961) showed that the primary cause of inactivation is carboxymethylation of the four methionine residues, resulting in unfolding of the RNase molecule (Stark *et al.*, 1961; Stark and Stein, 1964). Thus, the electron paramagnetic resonance spectrum due to reaction of RNase with I in 8 M urea, which is similar in form to that in Figure 3a, is undoubtedly due to labeling some or all of the methionine residues. The increased intensity of the electron paramagnetic resonance spectrum over that obtained under normal conditions indicates that more sites than usual have been labeled, and the greater mobility of the spin label indicates that the residues to which the spin label is attached are in a relatively open environment, consistent with an unfolding of some of the tertiary structure of RNase.

RNase Spin Labeled with the Bromo Amide Nitroxide II. Stark *et al.* (1961) found that iodoacetamide did not react with RNase at pH 5.5 in acetate buffer. Heinrickson (1966) found that at pH 8.5 iodoacetamide reacted with lysine-41 to yield mono- and dicarboxamidomethylated derivatives, and to a lesser extent

with lysine-1. In view of the increased reactivity of bromoacetate over iodoacetate as an alkylating agent for RNase, it might be anticipated that bromoacetamide would be more reactive than iodoacetamide. This speculation is confirmed by the observation that the bromo amide spin label II reacted rapidly with RNase at pH 5.5 and 8.5.

The amino acid analyses of RNase-II (Table I) indicate the presence of 3-carboxymethylhistidine (approximately 0.5 mole/mole of RNase A) and, to a lesser extent, ϵ-carboxymethyllysine (approximately 0.1 mole/mole of RNase A). Extensive studies (Stein, 1964; Crestfield *et al.*, 1963a,b; Heinrickson *et al.*, 1965; Heinrickson, 1966) have indicated that carboxymethylation of histidine-119 in RNase A takes place at the one position, whereas similar reaction with histidine-12 takes place at the three position. The two carboxymethylhistidine isomers emerge at different elution volumes (Crestfield *et al.*, 1963a,b) during the amino acid analysis and can be distinguished from one another. It is possible therefore to conclude that II reacted with histidine-12 of RNase A, and to a lesser extent with lysine residues.

RNase spin labeled with II at pH 8.5 gives results dissimilar to those for I. The broad components in the electron paramagnetic resonance spectrum (Figure 3b) are quite pronounced and the spectrum undergoes a change corresponding to further decrease in the mobility of the spin label when RNA is added. This implies that residues very near the site of substrate binding have been labeled (if allosteric effects are unimportant). The reaction of II with 1-CMHis-119-RNase at pH 5.5 or 8.5 yields an electron paramagnetic resonance spectrum with no broad component, which is insensitive to RNA addition. The amino acid analyses of Table I indicate that the labeling at pH 8.5 is much less specific than at pH 5.5, the major products being ϵ-carboxymethyllysine (0.6 mole/mole of RNase), ϵ-dicarboxymethyllysine (0.3 mole/mole of RNase), and 3-carboxymethylhistidine (0.2 mole/mole of RNase). By analogy with the results of Heinrickson (1966) using iodoacetamide at pH 8.5, we assert that lysine-41 has been labeled. As with label II at pH 5.5, it is possible to conclude with certainty that the 3-carboxymethylhistidine resulted from reaction of II with histidine-12 of RNase A. The presence of a narrow component in the electron paramagnetic resonance spectrum of RNase labeled with II at pH 5.5 and 8.5, and in that due to reaction of 1-CMHis-119-RNase at pH 5.5 and 8.5, indicates that some residue other than those at the active site has been labeled. The amino acid analyses show that these are lysine residues, and the electron paramagnetic resonance data indicate that these particular lysine residues are located on the surface of the protein.

The electron paramagnetic resonance spectra due to oxidized RNase labeled with II and oxidized RNase-II indicate that the spin label has a high degree of mobility. This is to be expected since oxidation of RNase removes a sufficient number of constraints to disrupt the complex tertiary structure.

Activity Measurements. The observation that the RNase-I and -II samples retain 50% of the specific activity of parent RNase indicates incomplete reaction rather than complete reaction with partial inactivation. This is because the experimental evidence shows that reaction with histidine residues at the active site of RNase has taken place, and it has been established that the carboxymethylhistidine derivatives of RNase have no enzymic activity (Heinrickson *et al.*, 1965). However, such a conclusion cannot be reached for the products with III and IV. The reactivities of III and IV toward RNase were much greater than those of I and II, and complete reaction might be expected. This question can of course be resolved by chromatographic isolation of the various spin-labeled derivatives. Such a program is under way, and the results will be reported at a later date.

Effect of RNA on Spin-Labeled RNase. The electron paramagnetic resonance spectra of Figure 4a,d indicate that upon addition of RNA the motion of the spin label responsible for the broader spectral components has been restricted even further. This could arise in two ways. (i) RNA and RNase form a *random* complex with a longer rotational correlation time than RNase itself, and no direct change in the environment of the spin label occurs. (ii) RNA and RNase form a *specific* complex resulting in a change in the immediate environment of the spin label at the active site. In the former case the effect is due to a change in the motion of the protein molecule as a result of complex formation; in the latter case the effect is mainly due to restriction of the motion of the spin label relative to that of the protein molecule. It is significant that addition of RNA to RNase-III, or to 1-CMHis-119-RNase labeled with I or II, *i.e.*, derivatives spin labeled only at the lysine residues on the exterior of the molecule, causes no observable change in the electron paramagnetic resonance spectra. Thus, it is most probable that the influence of RNA on the electron paramagnetic resonance spectra of RNase-I, -II, and -IV is due to the formation of a specific complex. The stability of the complex between spin-labeled RNase derivatives and RNA provides further evidence for the specific nature of the binding. Whether the observed immobilization of the spin label in the enzyme is due to a conformational change in RNase upon binding RNA, or due to direct contact between the label and the substrate, cannot be decided at this point. Further studies to explore this question are in progress.

Addition of RNA core or 2′-cytidylic acid to RNase-I or -II produced no change in the electron paramagnetic resonance spectra. This indicates that if the spin-labeled RNase does bind these species, it does so in a manner different to that for substrate RNA. This is to be expected in view of the observation by Yang and Hummel (1964) that whereas RNase A binds 2′-cytidylic acid in such a way as to alter the ultraviolet spectrum of the nucleotide, the carboxymethylated derivatives do not.

The formation of a specific complex between RNA and RNase inactivated by spin labeling demonstrates

that even though the final steps in the enzymic process are prevented by the chemical modification of a single amino acid residue, the earlier steps involving recognition and binding of appropriate substrates are not.

Effect of Added Polyribonucleotides. The substrate specificity of RNase A has been a controversial subject for some time (Scheraga and Rupley, 1962; Hummel and Kalnitsky, 1964). From the nature of the liberated oligonucleotides it appears that RNase hydrolyzes a $C_3'OP(O)(O)OC_5'$ linkage only if the C_3' end is part of a pyrimidine nucleotide (C or U). Thus, it is not surprising that RNase has been found to hydrolyze poly U and poly C (Michelson, 1958). However Beers (1960) showed that RNase A acts also on poly A and poly I, and Imura *et al.* (1965) found the mode of action to be similar to that with substrate RNA. As yet no positive data on interaction with poly G are available; in fact Michelson (1958, 1959) reported that RNase had no action on poly G.

The present data indicate that RNase A, inactivated by labeling one of the histidine residues of the active site, still binds strongly poly A, poly G, poly I, and poly C, but not poly U, in a manner similar to that with which it binds substrate RNA. The effects of poly A, poly I, and poly C are understandable in view of the known reactivity of RNase A toward them; the effect of poly G indicates that RNase A does bind it in a specific manner and suggests that under optimal conditions RNase A can hydrolyze poly G. The lack of a specific binding effect due to poly U is puzzling. It may be significant that, at the pH of these experiments, all the polynucleotides binding to RNase strongly enough to cause an electron paramagnetic resonance spectral change have highly ordered structures, whereas poly U does not. That is, the degree of spin-label constraint caused by binding a polynucleotide may be dependent on the secondary structure of the polynucleotide. This seems unlikely in view of the rather small amount of secondary structure thought to exist in RNA, but this possibility is currently under study using ribonucleotide oligomers of varying composition and chain length.

Effect of Urea on the Electron Paramagnetic Resonance Spectra of RNase Labeled with I, II, and III. The failure of a saturated urea solution to expand the structure of RNase-I (the bromo acid derivative) sufficiently to increase the mobility of the spin label to a detectable extent is striking. This implies that the spin label is capable of maintaining the conformation of the active site under the influence of severe disruptive forces and is consistent with the observation by Yang and Hummel (1964) that the rate of denaturation by 8 M urea of 1-CMHis-119-RNase, as measured by the normalization of the ultraviolet spectrum due to the buried tyrosine residues, is much slower than that of RNase A. The present study indicates that the immediate environment of the spin-labeled histidine residue *does not change noticeably in 8* M *urea* even after times sufficient for normalization of the tyrosine residues and demonstrates the care which must be exercised in using the observation of changes in the structure of one part of a biological macromolecule to predict similar changes in other parts not directly observable (Barnard, 1961). The failure of the bromo amide label II to prevent the expansion is doubtless due to lack of a carboxyl group to bind to a histidine residue at the active site. It is significant that the electron paramagnetic resonance spectrum of RNase labeled with II was essentially unchanged in 4 M urea, although it was drastically changed by 8 M and saturated urea, *i.e.*, the active site environment of RNase is essentially intact in 4 M urea. In the case of 1-CMHis-119-RNase-II the carboxymethyl substituent on His-119 would serve to hold the active site together in 8 M urea, as was observed for the bromo acid spin label I, and the positions of the spin labels on the exterior of the protein structure would make them much less sensitive to any structural change which may have occurred. The reversibility of the observed urea effects is in accord with previous reports that urea denaturation of RNase is reversible (Harrington and Schellman, 1956). Lack of any electron paramagnetic resonance spectral change on addition of urea to RNase-III provides further evidence for the assignment of the labeling sites to residues located on the exterior of the protein molecule.

It has been observed that RNase retains partial activity in 8 M urea in the presence of RNA (Anfinsen *et al.*, 1955), and suggested that binding of RNA helps maintain the particular structural arrangement necessary for enzymic activity (Sela *et al.*, 1957). Optical measurements have been consistent with this interpretation (Sela *et al.*, 1957). The present data for RNase-II show that in the presence of RNA the immediate environment of the active site expands in a manner quite similar to that in its absence. The slight broadening observed in the spectra indicates that interaction with RNA does occur, but to an extent involving much less constraint of the spin label at the active site. The complex formed between labeled RNase A and RNA in 8 M urea must be considerably weaker than that formed under normal conditions, since the residual RNase activity in the solution was sufficient to hydrolyze the added RNA in the time required to remove the urea by dialysis (under normal conditions the complex between RNase labeled with II and RNA is resistant to active RNase A).

Temperature Effects on Optical and Electron Paramagnetic Resonance Spectra. From optical studies it is known that RNase undergoes a reversible thermal transition with a characteristic melting temperature (Harrington and Schellman, 1956). At the pH of the present electron paramagnetic resonance experiments the melting temperature is approximately 61°. This optical behavior is thought to be due to three abnormal tyrosine residues which are "buried" in a hydrophobic environment (Li *et al.*, 1966). Over the range 5–45° no appreciable change in optical density has been observed (Hermans and Scheraga, 1961). Up to the present it has been accepted that the active site region of the RNase molecule responded to perturbations in a manner parallel to that of the abnormal tyrosine residues, and therefore the optical difference spectrum was taken as a

useful monitor of the integrity of the active site.

The present measurements of optical spectra and melting temperatures for the spin-labeled derivatives of RNase indicate that the labeling has caused only a very minor change in the forces maintaining the structure of the macromolecule and lend further credence to our thesis that the properties of spin-labeled RNase may be used to discuss the properties of the parent molecule.

The gradual mobilization of the spin labels in RNase-II and RNase-IV over the temperature range 5–35° indicates that the active site region of RNase A is undergoing a significant expansion. The rather small activation energy for the expansion process over the range 5–35°, 5.5 and 5.4 kcal/mole for RNase-II and -IV, respectively, is indicative of the breaking of only one or two hydrogen (or hydrophobic) bonds. The identity of the activation energies for two such different spin labels, attached to different components of the active site, argues against this energy being due to specific interaction between the protein and spin-label prosthetic groups. The crossover point at 35°, and the higher activation energies thereafter of 10.8 and 9.6 kcal/mole for RNase-II and RNase-IV, respectively, indicate that a second major step in the expansion of the region of the active site comes into play. It seems likely that this is the same process involved in the normalization of the tyrosine residues over the temperature range 45–65°. The somewhat greater ease with which label IV is mobilized over the higher temperature range is consistent with the current crystallographic data (Kartha *et al.*, 1967), which indicate that Lys-41 is in a less tightly packed region of the RNase molecule than His-12 or His-119. The very slight change in mobility of label III with increasing temperature provides further evidence for its being attached to residues located on the periphery of the protein molecule.

pH Effect. The reversibility of denaturation by low pH has been established previously (Hermans and Scheraga, 1961). The results with RNase-I and -II demonstrate that the structural change is more drastic than that induced by saturated urea; the appearance of the powder-type spectrum on reattaining pH 5.5 indicates that the structural change at low pH was sufficient for the spin label to reorient itself into a more favorable position. Whereas label I was able to prevent expansion of the active site by saturated urea, it was unable to do so in the case of low pH. The results obtained by denaturation at pH 11 are consistent with an irreversible denaturation process resulting in considerable disruption of the compact three-dimensional structure of ribonuclease. The lack of an observable change in the electron paramagnetic resonance spectrum of 1-CMHis-119-RNase spin labeled with II when the pH is varied over the range 5.5–2.5–5.5 provides further confirmation for the assignment of the labeled positions to lysine residues on the exterior of the protein structure. Irreversible narrowing of the electron paramagnetic resonance spectrum of this system when the pH is raised to 11 is indicative of the gross structural change involved in irreversible denaturation.

Spin Labeling with III and IV. All the results obtained by reaction of RNase with the maleimide spin label III are consistent with the assumption that III reacts mainly with lysine residues on the exterior of the protein molecule. The appearance of a broad component in the electron paramagnetic resonance spectrum of RNase-IV, which is sensitive to RNA addition, indicates that IV is able to react with an amino acid residue near the active site of the enzyme, presumably lysine-41. The greater mobility of the spin label in RNase-IV relative to RNase-II, and its weaker response to RNA addition, indicate that lysine-41 is on the periphery of the active site, and closer to the exterior of the protein molecule. This is in accord with the model for RNase reported by Kartha *et al.* (1967).

From the results of labeling RNase-III with II it appears that the usual reaction of II has taken place and that the immediate environment of label II is similar to that without prior labeling with III. The failure of RNA addition to effect the usual spectral change suggests that RNase-III is incapable of binding RNA.

RNase S-II and RNase S Protein-II. RNase S is an enzymically active noncovalent complex formed by the two fragments, RNase S protein and RNase S peptide, obtained by cleavage of RNase A between residues 20 and 21 with the enzyme subtilisin (Kalman *et al.*, 1955). Although retaining most of the properties of RNase A, RNase S appears to have a somewhat more open molecular structure (Ottesen and Stracher, 1960; Richards and Vithayathil, 1960; Vithayathil and Richards, 1960). The electron paramagnetic resonance spectrum of RNase S-II indicates that the spin label is slightly more mobile than in RNase-II, but not nearly as much as one might have expected on the basis of other spectroscopic data. Assuming that II has reacted with one of His-12 or His-119 in the active site of RNase S, it appears that the active site environments in RNase A-II and RNase S-II are very nearly the same. The similarity between the electron paramagnetic resonance spectra upon addition of RNA to either species corroborates this assertion.

The electron paramagnetic resonance spectrum of RNase S protein-II (similar to Figure 2d) indicates that the spin label is considerably more mobile than in RNase S-II. The resemblance between the spectra before and after RNA addition to those obtained with RNase-IV is striking. RNase S protein lacks His-12 and therefore cannot have an active site like that of RNase A or S. Thus, His-119 should not have enhanced reactivity toward the alkylating spin label, and it is likely that II would not react with histidine (II does not react with normal histidine residues in proteins: I. C. P. Smith and L. J. Berliner, unpublished data). The obvious choice for a reaction site is Lys-41, particularly in view of the similarity of the spectra to those obtained with RNase-IV. The positive RNA effect indicates that although RNase S protein lacks sufficient residues to constitute an active site, it does retain enough of the structure responsible for RNA recognition to bind RNA.

Conclusions

The bromo acid and bromo amide spin labels (I and II) react preferentially with a histidine residue at the active site of RNase A. They also react to a lesser extent with lysine residues located on the exterior of the protein molecule. The five-membered maleimide nitroxide label III reacts only with these peripheral lysine residues, whereas the six-membered maleimide nitroxide IV labels primarily a lysine residue near the active site, presumably lysine-41. The electron paramagnetic resonance data indicate that lysine-41 is in a more open portion of the RNase structure than are histidine-12 and histidine-119. It is possible to show that label II reacts preferentially with histidine-12, whereas a choice between the two possibilities for label I cannot be made at this point. The spin-labeled derivatives are very similar in structure to the parent enzyme, as indicated by their optical spectra and melting behavior.

Although spin labeling the histidine residues destroys enzymic activity in RNase A, it apparently does not interfere with the process by which the enzyme recognizes and binds suitable substrates.Even in the case of RNase S protein, which is inactive due to the lack of S peptide, sites responsible for recognition and binding of substrate still function. The active site environments of spin-labeled RNase A and S are much more similar than anticipated on the basis of other data.

The denaturation of RNase A caused by low pH produces a more severe disruption of the active site region than does saturated urea. High pH denaturation is the most severe, and is irreversible. Carboxyalkyl substituents on one of the histidine residues in the formerly active site can stabilize the structure against urea denaturation, but not against either denaturation by low or high pH, or thermal denaturation.

Spin-labeled RNase A can bind RNA in 8 M urea, but much more weakly than in the absence of urea, and the active site region is considerably less compact in this medium. If an enzyme–substrate fit does occur in 8 M urea, it is very different from the processes involved in the absence of urea. The active site environment of RNase-II is essentially unchanged by 4 M urea but greatly expanded by 8 M urea. Polyguanylic acid binds to spin-labeled RNase in a manner similar to that of RNA, polyadenylic acid, polycytidylic acid, and polyinosinic acid; polyuridylic acid does not.

The active site of spin-labeled RNase A expands with temperature over the region 5–45°, although this is not indicated by changes in the optical absorption of the tyrosine residues. Therefore, the abnormal tyrosine residues in RNase A do not constitute a monitor of the active site conformation.

Acknowledgments

It is indeed a pleasure to acknowledge the advice and support of Professor H. M. McConnell, in whose laboratory this study was begun. I am grateful to Dr. R. G. Shulman of Bell Telephone Laboratories for hospitality during 1966–1967 and to Professor G. R. Stark for advice and a critical reading of the manuscript. I am indebted to Dr. T. Yamane, Dr. S. Ogawa, and Dr. C. L. Hamilton for helpful discussions, to Professor L. M. Krausz for performing the amino acid analyses, to Professor F. M. Richards for samples of RNase S and RNase S protein, to Dr. T. Schleich for measuring the thermal–optical profiles and for his constructive criticism of the manuscript, to Dr. W. C. Landgraf for compound IV, and to Mrs. R. C. Dawkins for technical assistance.

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