charged polycations, and the electrostatic repulsions between the numerous positive charges located at their surfaces may well contribute to their destabilization and consequent unfolding. In cytochrome *c*-552 the corresponding destabilization phenomenon should be much weaker since there could exist at low pH no more than 6 positive charges distributed over a similar area (Pettigrew, 1974). The persistence of the native closed-crevice structure at acid pH, as visualized by the absence of spectral changes, indicates also that the degree of ionization of the carboxyl groups does not affect the folding of the chain around the heme.

Addition of neutral salts to horse cytochrome *c* unfolded by acid results in partial refolding, dependent on the nature of the anion and its concentration (Aviram, 1973; Lanir & Aviram, 1975). The stability of cytochrome *c*-552 in solutions of HCl would reflect a relatively higher affinity for chloride. Since, however, chloride is the weakest of a series of anions effective in stabilization of horse cytochrome *c*, this hypothesis can be experimentally tested only by direct binding experiments.

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Spin-Labeled Ribonuclease A. Effects of Chemical, Enzymatic, and Physical Modifications on Enzyme Conformation†

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ABSTRACT: 3-SLHis-105-RNase A is an active derivative of ribonuclease A (RNase A) spin-labeled at the 3 position of the imidazole ring of histidine-105. The spin-labeled enzyme has been modified by urea denaturation, reduction, reduction–carboxymethylation, performic acid oxidation, and digestion with proteolytic enzymes in order to monitor changes in the geometry of the protein by changes in the electron paramagnetic resonance (EPR) spectrum of the nitroxide spin-label probe. The results of these experiments indicate that the spin-label attached to histidine-105 of RNase A is sensitive to modifications affecting the conformational integrity of the molecule and to the reconstituting effects of various active-center ligands.

Proteins are dynamic macromolecules whose conformational properties relate closely to biological activity. Consequently, many studies of proteins have involved investigation of their conformational properties. Spin-labeling, or labeling a molecule with a stable organic free radical, has been introduced (Stone et al., 1965; Ohnishi and McConnell, 1965) as a possible means of measuring subtle structural changes in biological macromolecules. Reaction of bovine pancreatic ribonuclease A (RNase A) with the nitroxide spin-label *N*-(2,2,5,5-tetramethyl-3-pyrrolidinyl-1-oxy)bromoacetamide at pH 5.5 or 6.5 results in alkylation at the 3 position of the imidazole ring of histidine-105 (Daniel et al., 1973). The 3-SLHis-105-RNase A,[1] ~85% as active as RNase A, is inactivated by iodoacetate

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[1] Abbreviations used are: EPR, electron paramagnetic resonance; RNase A, ribonuclease A; 3-SLHis-105-RNase A or SL-RNase A, ri-

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at the same rate as RNase A and exhibits the same behavior as RNase A upon affinity chromatography. The present paper reports our use of spin-labeling to assess the conformational changes caused by several chemical and enzymatic modifications of SL-RNase A.

Experimental Procedure

Materials

The spin-labeling of bovine pancreatic ribonuclease A (EC 2.7.7.16) at histidine-105 with a bromoacetamide nitroxide spin-label has been described (Daniel et al., 1973). Pepsin (EC 4.4.1) was a product of Pentex. Subtilopeptidase A (bacterial protease, type VII) and substrates and inhibitors for RNase A were purchased from Sigma Chemical Co. Carboxypeptidase A (EC 3.4.2.1) (COADFP) and trypsin (EC 3.4.4.4) (TRPCK) were obtained from Worthington Biochemical Corp. Sephadex and Bio-Rex 70 resins were products of Pharmacia Fine Chemicals and Bio-Rad Laboratories, respectively.

Methods

Electron paramagnetic resonance (EPR) measurements were made using a JEOLCO JES-ME-3X resonance spectrometer at 9.5 GHz (X band) with 100-kHz modulation. Sample temperatures were controlled to 25 ± 1 °C by means of a temperature-regulating accessory supplied with the EPR instrument. The mobility of the spin-label was assessed by calculation of the ratio of the net heights of the first two principal lines in the EPR spectrum (*a/b* ratio) or by calculation of the effective rotational correlation time, τ_c^{eff}, according to the method of Stone et al. (1965). Amino acid analyses were carried out on a Beckman Model 110 amino acid analyzer. Trypsin digests were performed utilizing a Radiometer titrator for maintenance of constant pH. RNase A activity was determined by the spectrophotometric method of Crook et al. (1960).

Performic Acid Oxidation and Reduction–Carboxymethylation of RNase A and Derivatives. Oxidation of the disulfide bridges of RNase A, SL-RNase A, and enzymatically modified derivatives was performed at 0 °C according to the procedure of Harrington and Schellman (1965). Reduced-carboxymethylated (RCM) SL-RNase A was prepared as previously described (Daniel et al., 1973).

Reduction–Refolding of SL-RNase A. Approximately 5 mg of SL-RNase A was reduced with β-mercaptoethanol according to the method of Anfinsen and Haber (1961). As observed for reduced RNase A (Anfinsen and Haber, 1961), the reduced form of SL-RNase A was stable for several days at pH 3.5, 5 °C.

For studies of the effects of inhibitors on the structure of reduced SL-RNase A, the desalted, reduced protein was lyophilized from 0.1 N acetic acid following Sephadex chromatography (Hantgan et al., 1974). The reduced lyophilized protein was then dissolved in degassed 0.1 N acetic acid at a concentration of 0.2 mM. Samples from this solution on ice were then diluted 1:1 with degassed 0.3 N acetic acid containing 2 mM EDTA (pH 6.5), 0.3 M sodium acetate containing 2 mM EDTA (pH 6.5), 0.3 M sodium phosphate containing 2 mM EDTA (pH 6.5), or 0.2 M sodium acetate containing 0.10 M 2′-CMP and 2 mM EDTA (pH 6.5). The pH of each solution was measured and readjusted, if necessary, prior to flushing with nitrogen.

The refolding of reduced SL-RNase A (0.05 mg/mL) via air oxidation was carried out in 0.1 M ammonium bicarbonate, pH 8.5, for 29 h. The recovery of activity was monitored by withdrawing samples for enzymatic assay. The lyophilized reaction mixture was dissolved in 1.0 mL of 0.266 *m* sodium chloride and chromatographed on the Bio-Rex 70–NaCl system (1.0 × 20 cm column) of Crestfield (1963). Two peaks, one at 15 mL and the other at 98 mL, were detected by absorbance at 210 nm. Enzymatic activity was detected only at 98 mL. Active reoxidized SL-RNase A and the inactive component of the reaction mixture were desalted by Sephadex G-25 chromatography.

Digestion of SL-RNase A with Subtilopeptidase A. SL-RNase A was prepared as described by Doscher and Hirs (1967) for the preparation of RNase S. S-peptide and SL-S-protein were separated by Sephadex G-75 chromatography with 50% acetic acid as the eluent (Gross and Witkop, 1966). SL-RNase S′ was prepared by combining the S-peptide and SL-S-protein in a 1:1 ratio based on an activity vs. mole ratio plot.

Limited Pepsin Cleavage and Further Carboxypeptidase A Degradation of SL-RNase A. The preparation of des-(121–124)-SL-RNase A (SL-RNase P) was accomplished by limited digestion of SL-RNase A with pepsin as Anfinsen (1956) has described for RNase A. After 15 min of pepsin digestion at 37 °C, RNase A activity had diminished by 80%; 100 μL of 1.0 M sodium phosphate (pH 6.47) was then added to the 0.5-mL digest mixture. The resulting solution was chromatographed on a column (1.0 × 53 cm) of Bio-Rex 70 (−400 mesh) with 0.2 M sodium phosphate, pH 6.47, as the eluent. SL-RNase P was desalted by Sephadex G-25 chromatography, and characterized by enzymatic activity, amino acid analysis, and Sephadex G-75 chromatography subsequent to performic acid oxidation in order to establish that no peptide bonds, other than that between residues 120 and 121, had been hydrolyzed.

Phenylalanine-120 and histidine-119 were subsequently removed from SL-RNase P by carboxypeptidase digestion according to the conditions of Lin (1970). The resulting spin-labeled proteins were characterized by amino acid analysis.

Digestion of RNase A and SL-RNase A with Trypsin. Trypsin digests were performed essentially according to Ooi et al. (1963) and Ooi and Scheraga (1964a). Tryptic derivatives of RNase A, prepared for control purposes, were identified by amino acid analysis, enzymatic activity, and amino acid analysis of protein and peptide components following performic acid oxidation and Sephadex G-75 chromatography in 50% acetic acid.

Preparation of des-(32–33)-SL-RNase A and SL-RNase Tr (SL-RNase A cleaved between residues 31 and 32 or 33 and 34) was accomplished by digestion of SL-RNase A (10 mg/mL in 0.01 M KCl, pH 6.5) for 10 min; 5, 5, and 2 μg of trypsin were added at 0, 4, and 8 min, respectively, for every 45 mg of SL-RNase A present. SL-RNase Tr and des-(32–33)-SL-RNase A were isolated following Sephadex G-75 chromatography and Bio-Rex 70 (−400 mesh) chromatography as described by Ooi et al. (1963); each protein was then rechromatographed on a column (0.9 × 55 cm) of Bio-Rex 70 (−400 mesh) equilibrated with 0.2 M sodium phosphate, pH 6.47. Preparation of des-(34–37)-SL-RNase A and des-(32–37)-SL-RNase A was accomplished by digestion of

bonuclease A spin labeled at the 3 position of the imidazole ring of histidine-105; SL-RNase P, des-(121–124)-SL-RNase A; RNase S or SL-RNase S, RNase A or SL-RNase A cleaved between amino acid residues 20–21 or 21–22; RNase Tr or SL-RNase Tr, RNase A or SL-RNase A cleaved between amino acid residues 31–32 or 33–34; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

SL-RNase Tr (1 mg/mL in 0.01 M KCl, pH 7.8) with trypsin (0.04 mg/mg SL-RNase Tr) for 35 min at 21 °C; each protein was isolated following Sephadex G-75 chromatography and Bio-Rex 70 (−400 mesh) chromatography as described by Ooi and Scheraga (1964a). Each tryptic digest product isolated was identified by amino acid analysis.

Urea Denaturation–Renaturation of SL-RNase A and Tryptic Derivatives. SL-RNase A was denatured in a freshly prepared 8 M urea solution (pH 7.3) containing 0.01 M Tris. Renaturation was achieved by diluting the urea to 0.8 M with 0.01 M Tris (pH 7.3). A study of the inhibitor-induced renaturation of SL-RNase A was performed by first denaturing the protein (1 mM) in 8 (pH 6.5 or 5.5) or 9 M urea (pH 6.5) and then diluting samples of the denatured protein to 0.1 mM with urea solutions (at the same concentrations and pH) containing 0.1 M sodium acetate, 0.1 M sodium phosphate, 0.055 M cytidine 2′-monophosphate (2′-CMP), or 0.055 M 2′,3′-cyclic cytidylic acid. All samples were stored overnight at 5 °C so that EPR spectra could be recorded 24 h after sample preparation.

SL-RNase Tr and des-(32–33)-SL-RNase A were each denatured in a freshly prepared solution of 8 M urea (pH 6.5) containing 0.05 M sodium acetate. Renaturation was accomplished by dilution of the urea with 0.05 M sodium acetate (pH 6.5) and gel filtration on a column (0.9 × 57 cm) of Sephadex G-75 equilibrated with 0.05 M ammonium acetate.

Results

Spin-labeled RNase A was modified by urea denaturation, disulfide bond cleavage, heat denaturation, and digestion with pepsin, carboxypeptidase A, subtilopeptidase A, and trypsin. SL-RNase A and the spin-labeled derivative obtained by these modifications were studied by EPR both in the presence and absence of inhibitors. Reproducibility in determination of *a/b* ratios from different spectra of the same sample was approximately 2%; reproducibility in determination of τ_c^{eff} values from different spectra of the same sample was 3–4%.

N-(2,2,5,5-Tetramethyl-3-pyrrolidinyl-1-oxy)bromoacetamide in water has a spectral *a/b* ratio of 1.0 and τ_c^{eff} of 4.9×10^{-11} s. Covalent attachment of the spin-label to histidine-105 of RNase A causes a marked decrease in the mobility of the nitroxide; the spin-labeled enzyme in 0.1 M sodium acetate, pH 5.5, has a spectral *a/b* ratio of 0.4 and τ_c^{eff} of 2.3×10^{-9} s (for spectra, see Daniel et al., 1973). The line shape of the EPR spectrum of SL-RNase A could not be duplicated by increasing the viscosity of a solution of the free nitroxide (by variation of temperature and percent glycerin). Furthermore, we were unable to computer simulate the EPR spectrum of the spin-labeled protein by the method of Freed et al. (1971) and Goldman et al. (1972); these results imply that the motion of the attached nitroxide is more complex than any of the three rotational models incorporated in the computer program.

The spectrum of SL-RNase A in 8 M urea and 0.01 M Tris (pH 7.3) indicates that the spin-label is markedly more mobile when the enzyme is denatured in urea (*a/b* ratio = 0.7). The EPR spectrum of the same sample, diluted 1:10 with the Tris buffer, indicates that the structural changes in the region of the spin-label are reversible (*a/b* ratio = 0.5).

Results of the EPR studies of SL-RNase A in 8 M urea containing sodium acetate, sodium phosphate, or 2′-CMP are summarized in Figure 1a–d. SL-RNase A in 8 M urea, pH 6.5, has a spectral *a/b* ratio = 0.7. The presence of 0.10 M sodium phosphate in the urea solution causes a decrease in the *a/b* ratio to 0.6, whereas the presence of 0.10 M sodium acetate has little effect on this parameter (Figure 1a–c). Increasing the sodium

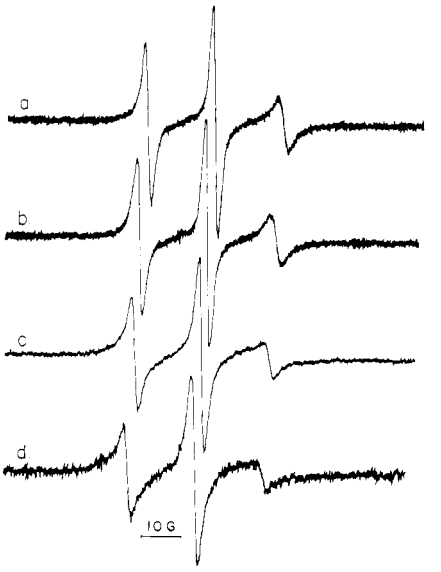

FIGURE 1: EPR spectra of SL-RNase A in (a) 8 M urea, pH 6.5; (b) 8 M urea, 0.1 M sodium acetate, pH 6.4; (c) 8 M urea, 0.1 M sodium phosphate, pH 6.5; (d) 8 M urea, 0.03 M sodium acetate, 0.05 M 2′-CMP, pH 6.5.

phosphate concentration to 0.15 M in 8 M urea, pH 6.5, causes no further change in the observed EPR spectrum (*a/b* ratio, 0.6). The presence of 0.05 M 2′-CMP in an 8 M urea solution (pH 6.5) of the spin-labeled enzyme causes the most dramatic effect; the *a/b* ratio decreases to 0.5 and the third line in the spectrum appears markedly broadened (Figure 1d). Furthermore, each inhibitor-induced change was found to be time dependent, the entire change taking place within 1–2 h after sample preparation; no further changes were evident when the EPR spectra were recorded 24 h later. Addition of the substrate 2′,3′-cyclic cytidylic acid to urea-denatured SL-RNase A (pH 6.5) causes no observable change in the EPR spectrum within a few hours of sample preparation.

Addition of orthophosphate (0.1 M) to unfolded SL-RNase A in 8 M urea, pH 5.5, causes little change in the EPR spectrum as recorded before the inhibitor is added (*a/b* ratio = 0.7); the addition of 2′-CMP (0.05 M) to unfolded SL-RNase A (pH 5.5) causes some decrease in the *a/b* ratio (0.7 to 0.6), but the effects are not as pronounced as those observed at pH 6.5. The results of an experiment in which the concentration of urea was 9 M (pH 6.5) indicate that the inhibitor-induced refolding of SL-RNase A is as extensive as that observed when the concentration of urea is 8 M (pH 6.5).

The disulfide bridges of SL-RNase A were cleaved by reduction, reduction followed by carboxymethylation, and performic acid oxidation. Figure 2a and Table I summarize the effects of these modifications on the EPR spectrum of the spin-labeled enzyme. In each instance, the spectral lines are much sharper, indicating a drastic increase in the mobility of the spin-label.

Refolding of reduced SL-RNase A was accomplished by air oxidation; the EPR spectra of active and inactive spin-labeled enzyme products, separated by chromatography of the salt-free oxidized mixture, are illustrated in Figure 2b,c. The active fraction, eluting from the Bio-Rex 70 column at the same position as SL-RNase A, exhibits an EPR spectrum (Figure 2b) identical to that of SL-RNase A. The activity of the purified, reduced, and refolded SL-RNase A was 100% of the original specific activity of the spin-labeled enzyme. The EPR spectrum of the inactive fraction (Figure 2c) is unlike that of SL-RNase A.

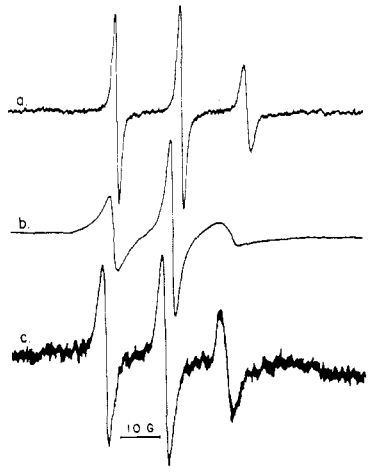

FIGURE 2: EPR spectra of (a) reduced SL-RNase A in 0.1 N acetic acid, (b) active, reduced-air oxidized SL-RNase A, and (c) inactive, reduced, air-oxidized SL-RNase A.

TABLE I: Spectral *a/b* Ratios of Oxidized and Reduced SL-RNase A.

Modified SL-RNase A	*a/b* ratio
Performic acid oxidized in H_2O	0.9
Reduced and carboxymethylated in H_2O	0.8
Reduced in 0.1 N HOAc	0.9
Reduced in 0.2 M acetate, 1 mM EDTA, pH 6.5	0.9
Reduced and air oxidized in H_2O	0.4

The addition of inhibitors to reduced, spin-labeled RNase A caused no apparent refolding (*a/b* ratio = 0.9) under conditions (0.1 mM enzyme, pH 6.5 in the presence or absence of 0.15 M sodium phosphate or 0.05 M 2′-CMP) where inhibitor-induced refolding of urea denatured SL-RNase A was marked.

The effects of heat denaturation on the EPR spectrum of SL-RNase A were also studied. At 65 °C the *a/b* ratio is 0.9 and τ_c^{eff} is 4.0×10^{-10} s. This dramatic increase in the mobility of the spin-label has been observed independently by Matheson et al. (1977), who prepared 3-SLHis-105-RNase A according to our method. Comparison of spectra obtained at 65 °C with spectra of reduced, spin-labeled RNase A at 25 °C or with spectra of urea denatured SL-RNase A (where solution viscosity is much greater) at 25 °C is of course not valid. The important observation is that on lowering the temperature to 25 °C the thermally induced changes reverse. This result is consistent with data by Harrington and Schellman (1956), indicating that RNase A undergoes a reversible unfolding transition when heated.

The structure of SL-RNase A was modified by enzymatic degradation in three different regions. Table II summarizes the results of the studies of these enzymatically modified spin-labeled proteins. Preparation of SL-RNase S was accomplished by digestion of SL-RNase A with subtilopeptidase A; SL-RNase S was subsequently separated into SL-S-protein and S-peptide by gel filtration. The EPR spectra of SL-RNase S and SL-S-protein are shown in Figure 3a,b. SL-RNase S has an *a/b* ratio of 0.5; removal of S-peptide results in a substantial increase in the *a/b* ratio from 0.5 to 0.7.

Recombination experiments showed a 1:1 stoichiometry for recombination of SL-S-protein and S-peptide; both SL-RNase S and SL-RNase S′ had an enzymatic activity equal to that of SL-RNase A (~85% of the activity of RNase A). Other

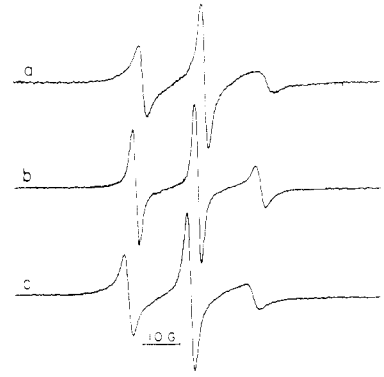

FIGURE 3: EPR spectra of (a) SL-RNase S in 0.1 M sodium acetate, pH 5.5; (b) SL-S-protein in 0.1 M sodium acetate, pH 5.5; (c) SL-S-protein in 0.05 M sodium acetate, 0.05 M 3′-CMP, pH 5.5.

TABLE II: Spectral *a/b* Ratios of Enzymatically Modified SL-RNase A.

	Buffer or inhibitor added			
Enzyme	Acetate[a]	Phosphate[b]	2′-CMP[c]	3′-CMP[d]
SL-RNase A	0.4[e]	0.4[e]	0.4[e,f]	0.4[e,f]
SL-RNase P	0.5	0.5	0.5	0.5
Des-(120–124)-SL-RNase A	0.6		0.5	0.5
Des-(119–124)-SL-RNase A	0.6		0.6	
SL-RNase S	0.5	0.5	0.5	0.5
SL-S-protein	0.7	0.6		0.5
SL-RNase Tr	0.5[g]	0.4[h]	0.5[g]	

[a] 0.1 M NaOAc, pH 5.5. [b] 0.2 M sodium phosphate, pH 5.5. [c] 0.05 M 2′-CMP, 0.05 M NaOAc, pH 5.5. [d] 0.05 M 3′-CMP, 0.05 M NaOAc, pH 5.5. [e] 0.4 M also at pH 6.5. [f] pH 5.4. [g] 0.5 M also at pH 6.5. [h] pH 6.5.

recombination experiments showed that either S-peptide, derived from RNase S, activated SL-S-protein, or S-peptide, derived from SL-RNase S, activated S-protein from RNase S. The EPR spectrum of SL-RNase S′ appears identical to that of SL-RNase S (*a/b* ratio = 0.5). The effects of adding inhibitors to SL-RNase S and SL-S-protein are summarized in Table II and Figure 3c.

Degradation of the C-terminal of SL-RNase A was accomplished with pepsin followed by carboxypeptidase A digestion. The EPR spectra of SL-RNase P and des-(120–124)-SL-RNase A are shown in Figure 4a,b. Removal of the tetrapeptide (121–124) from SL-RNase A results in an increase in the *a/b* ratio from 0.4 to 0.5, concomitant with a sharpening of the third principal line of the spectrum. Removal of phenylalanine-120 from SL-RNase P results in a further increase in the *a/b* ratio to 0.6. However, removal of histidine-119 from des-(120–124)-SL-RNase A results in a spectral *a/b* ratio that is approximately the same as that observed for des-(120–124)-SL-RNase A. The effects of adding inhibitors to SL-RNase P, des-(120–124)-SL-RNase A, and des-(119–124)-SL-RNase A are summarized in Table II.

Preparation of RNase Tr, des-(32–33)-RNase A, des-(34–37)-RNase A, and des-(32–37)-RNase A, carried out for control purposes, was accomplished by digestion of RNase A and then RNase Tr with trypsin; these modified proteins had 27, 8, 7, and 7%, respectively, of the activity of RNase A toward 2′,3′-cyclic cytidylic acid. Preparation of SL-RNase Tr, des-(32–33)-SL-RNase A, des-(34–37)-SL-RNase A, and des-(32–37)-SL-RNase A was accomplished by following the

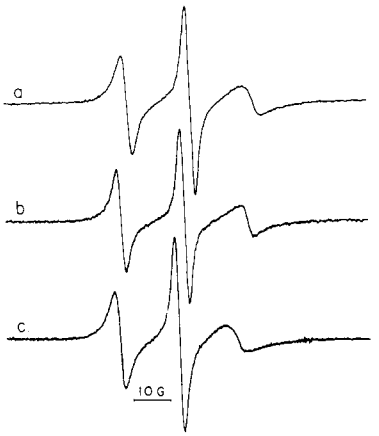

FIGURE 4: EPR spectra of (a) SL-RNase in 0.1 M sodium acetate, pH 5.5; (b) des-(120–124)-SL-RNase A in 0.1 M sodium acetate, pH 5.5; (c) SL-RNase Tr in 0.1 M sodium acetate, pH 6.5.

same procedure as for RNase A; however, the substrate–trypsin ratio was higher as SL-RNase A and SL-RNase Tr were slightly more susceptible to trypsin. The elution profiles (Bio-Rex 70) of the digests of SL-RNase A and SL-RNase Tr were quite similar to those tryptic digests of RNase A and RNase Tr.

The EPR spectrum of each tryptic digest product is similar in appearance to the spectrum of SL-RNase A; however, in each case the *a/b* ratio is greater, indicating that the spin-label is more mobile. The *a/b* ratios are 0.5 for SL-RNase Tr, des-(32–33)-SL-RNase A, des-(34–37)-SL-RNase A, and des-(32–37)-SL-RNase A. The EPR spectrum of SL-RNase Tr is shown in Figure 4c. Table II summarizes the effects of adding inhibitors to SL-RNase Tr. Addition of inhibitors to des-(32–33)-SL-RNase A and des-(32–37)-SL-RNase A caused very little change in the EPR spectra (data not shown).

Denaturation of SL-RNase Tr in 8 M urea containing 0.05 M sodium acetate (pH 6.5) results in a considerable increase in the mobility of the spin-label (*a/b* ratio = 0.7). Upon removal of the urea, the EPR spectrum (*a/b* ratio = 0.5) appears identical to that of SL-RNase Tr before denaturation. SL-RNase Tr was also examined for refolding in 8 M urea containing 0.15 M sodium phosphate or 0.05 M 2′-CMP (pH 6.5). No inhibitor-induced refolding was apparent from EPR spectra of these samples. Des-(32–33)-SL-RNase A was also denatured in 8 M urea containing 0.05 M sodium acetate, pH 6.5 (*a/b* ratio = 0.7). Removal of the urea yields a renatured enzyme whose EPR spectrum (*a/b* ratio = 0.5) is identical to that of des-(32–33)-SL-RNase A before denaturation.

Discussion

The chemical, structural, and dynamic aspects of RNase A have been described in depth over the last decade; the properties of the spin-labeled derivative, 3-SLHis-105-RNase A, appear similar to those of the native enzyme. We have modified SL-RNase A in several ways in order to more fully characterize the resulting structural changes by analyzing associated changes in the EPR spectrum of the nitroxide spin-label probe. When comparing spectral characteristics, the solvent conditions, temperature, and enzyme concentrations were carefully controlled, since minimal variations in these parameters (e.g., solution viscosity) were found to alter the spectral line shape.

The chemical modifications of SL-RNase A studied in this investigation include urea denaturation, performic acid oxidation, reduction, and reduction–carboxymethylation. The EPR studies of urea-denatured SL-RNase A indicate that the structural changes in the region of the spin-label are reversible. These results are consistent with the observations of Harrington and Schellman (1956), indicating the reversibility of the urea denaturation of RNase A. Furthermore, addition of orthophosphate or 2′-CMP to the urea-denatured spin-labeled enzyme (pH 6.5) causes decreased mobility of the spin-label. These spectral changes demonstrate that inhibitor-induced organization of the enzyme structure to a more compact form can occur. Indeed, Sela and Anfinsen (1957) and Sela et al. (1957), on the basis of spectrophotometric and polarimetric measurements, have shown that the physical changes caused by dissolving RNase A in 8 M urea, pH 6.5, could be substantially prevented or reversed by the presence of certain polyvalent anions. Barnard (1964a,b) also observed phosphate-induced refolding of urea-denatured RNase A. The effects of adding orthophosphate or 2′-CMP to urea-denatured SL-RNase A at pH 5.5 are not as pronounced as those at pH 6.5. Thus, the inhibitor-induced changes occur more readily at the higher pH, possibly because there are fewer enzyme charge–charge repulsions.

Studies of reduced, reduced–carboxymethylated, and performic acid oxidized SL-RNase A suggest that substantial unfolding of the enzyme takes place when the restraining effects of the disulfide bridges are removed. These results are consistent with the earlier intrinsic viscosity studies of Harrington and Schellman (1956), which demonstrated that near total disruption of RNase A tertiary structure results from cleavage of the disulfide bonds of the protein with performic acid. We have been unable to detect a change in the appearance of the EPR spectrum of reduced SL-RNase A with the addition of sodium phosphate or 2′-CMP. Since the EPR spectrum of reduced SL-RNase A represents all of the molecules in solution, one cannot rule out the possibility that some of the reduced molecules are partially refolded.

The enzymatic modifications of SL-RNase A include pepsin, carboxypeptidase A, subtilopeptidase A, and trypsin digestion. EPR studies of SL-RNase S indicate that cleavage of SL-RNase A with subtilopeptidase A causes an increase in the mobility of the spin-label. The appearance of the spectrum reveals, however, that only a small alteration in the structural integrity of the enzyme occurs. This result is consistent with results obtained from studies of RNase S. RNase S is similar to RNase A in regards to spectrophotometric titration of tyrosine residues (Richards and Vithayathil, 1960) but, unlike RNase A, is very susceptible to tryptic digestion (Richards and Vithayathil, 1959).

Separation of S-peptide from SL-S-protein results in a pronounced increase in the mobility of the spin-label; this parallels the observation of Richards and Logue (1962) that S-protein derived from RNase S contains two more "normally" titrating tyrosine residues than RNase A. Furthermore, our spectral data and activity assays indicate that SL-RNase S, like RNase S (Richards and Vithayathil, 1959), can be reversibly dissociated to protein and peptide components.

Addition of phosphate or 3′-CMP to SL-S-protein causes dramatic decreases in the mobility of the spin-label, suggesting that the SL-S-protein can bind to these inhibitors and in so doing folds around them to achieve a more nativelike conformation. These results are pertinent to the data of Woodfin and Massey (1968), who found that the presence of substrate alters the affinity of binding between S-protein and S-peptide.

When limited pepsin digestion removes the C-terminal tetrapeptide from SL-RNase A, a moderate increase in the mobility of the spin-label results. The mild spectral changes indicate that a large alteration in the structural integrity of the

enzyme does not occur despite the drastic loss in enzymatic activity. Lin (1970) found that the K_m of RNase P was twice that of RNase A and attributed the 99.5% activity loss to an increase in the pK of histidine-119. Puett (1972), on the basis of circular dichroism data, suggested that, although RNase P is less stable than RNase A, the conformations of the two molecules are similar. Our results support Puett's conclusion.

Removal of phenylalanine-120 from SL-RNase P promotes another significant increase in the mobility of the spin-label, a result compatible with the proposed importance of phenylalanine-120 in maintaining the structural stability of the C-terminus of RNase A (Lin, 1970; Gutte et al., 1972). Des-(120–124)-SL-RNase A also retains enough tertiary structure to bind inhibitors, further evidence for the subtle nature of the conformational changes brought about by these C-terminal degradations. Removal of histidine-119 from des-(120–124)-SL-RNase causes little change in the observed EPR spectrum, results consistent with data by Lin (1970) regarding the corresponding derivatives of RNase A.

EPR studies of SL-RNase Tr, des-(32–33)-SL-RNase A, des-(34–37)-SL-RNase A, and des-(32–27)-SL-RNase A indicate that all four proteins are conformationally similar in the region of the spin-label. Perhaps the label is too distant from the modified region to detect minor local conformational differences between these proteins. The EPR spectrum of each of the tryptic digest products is also similar in appearance to the spectrum of SL-RNase A, although the mobility of the spin-label in each instance is increased. Spectral data from other experiments indicate that the urea-induced denaturation of SL-RNase Tr and des-(32–33)-SL-RNase A is reversible following removal of the urea. Ooi and Scheraga (1964b) found that RNase Tr (designated component IV), des-(32–33)-RNase A, and des-(32–37)-RNase A all had three "abnormally" titrating tyrosine residues, as does RNase A. Based on this and thermal transition temperature data, these workers concluded that the structures of the tryptic derivatives were similar to that of RNase A but more flexible.

The studies reported here and the studies of Ooi and Scheraga (1964a,b) indicate that the drastic losses in activity associated with modification of RNase A between half-cystines-26 and -40 result from modification of an important area of the molecule and not from disruption of the conformation of the entire molecule. Lysine-41, implicated as important to the activity of RNase A (Richards and Wyckoff, 1971), is near the area modified by trypsin.

The covalent attachment of a spin-label probe to histidine-105 of RNase A has given us the opportunity to assess protein conformational changes, formerly evaluated with rather indirect methods (e.g., change in intrinsic viscosity, UV spectrum, thermal melting profile, enzymatic activity, chemical reactivity, and enzymatic susceptibility), by a more direct and general method. Since spin-labels are useful as conformational probes of molecular structure only in cases where the monitored structural change occurs near the label or is transmitted to its environment through the molecule, one might expect such labels to be more useful in proteins without the structural "rigidity" (Anfinsen and Scheraga, 1975) afforded by disulfide bridges. Despite the stabilizing influence of the four disulfide bridges in RNase A, substantial freedom exists within the molecule to monitor the effects of the various structural modifications investigated here. Perhaps many of the EPR line-shape changes would have been greater had the nitroxide been located closer to the active site, but a large perturbation of enzyme function might also have resulted. Nevertheless, the EPR results presented here are consistent with the results of previous studies of RNase A and, in general, support current concepts of the dynamic nature of proteins in solution.

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