ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY AS A MONITOR OF THE PATHWAY OF THE THERMAL UNFOLDING OF RIBONUCLEASE A

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SUMMARY: Two compounds were used to label residues His-105 and Lys-1, respectively, of ribonuclease A. Comparison of the temperature dependence of the rotational correlation times of the two differently located spin labels indicates a considerable difference between the temperatures at which separate regions of the protein molecule exhibit conformational mobility. These observations support the concept that the pathway of the thermal unfolding of ribonuclease A is sequential.

We are using the spin-labeling technique (1-3) to investigate the pathway of folding of bovine pancreatic ribonuclease. Here, we report the results of two such labeling experiments. One spin label is attached to His-105, and the other is probably attached to the N-terminal α-amino group; comparison of their respective correlation times as ribonuclease is denatured thermally reveals substantial differences in the temperature of unfolding of the C- and N-termini of the molecule.

MATERIALS AND METHODS

Worthington bovine pancreatic ribonuclease A was purified by the method of Taborsky (4). Trypsin and aminopeptidase were obtained from Worthington. 3-(2-Bromoacetamido)-2,2,5,5-tetramethyl pyrrolidine-1-oxyl (BSL) was purchased from Syva Corp. The N-hydroxysuccinimide ester of 3-carboxy-2,2,5,5-tetramethyl pyrrolidine-1-oxyl (HSL) was prepared by the procedure of Andersson and Fölsch (5). Column chromatography was carried out with Sephadex G-25 (Pharmacia), Bio-Rex 70 (-400 mesh, Bio Rad) and Dowex 50W-X2 (Bio Rad). Buffer solutions were made with the appropriate chemicals of the purest form available in distilled deionized water.

BSL was reacted with ribonuclease A by stirring 3-4 mg of the spin label together with 17-18 mg of enzyme in 1.0 ml of 0.2 M sodium acetate buffer (pH 5.5) for 110 hrs in the dark at room temperature. The HSL spin label was added as a suspension (30 µl of 50 mg/0.4 ml dioxane suspension) to a 1.0 ml solution of ribonuclease A (22 mg) in the same acetate buffer. The pH of this reaction mixture was quickly adjusted to 8.0 with 5% KOH, and the solution stirred in

Abbreviations: BSL, 3-(2-bromoacetamido)-2,2,5,5-tetramethyl pyrrolidine-1-oxyl; HSL, 3-carboxy-2,2,5,5-tetramethyl pyrrolidine-1-oxyl.

the dark at room temperature for 110 hrs. In both cases, the labeling reaction was stopped by desalting on a 3.3 x 74 cm column of Sephadex G-25 with 5% acetic acid. Fractions containing protein were lyophilized, taken up into 0.2 M sodium phosphate buffer (pH 6.5) and chromatographed on a Bio-Rex 70 column (1.0 x 13.0 cm) according to Daniel et al. (6).

The electron paramagnetic resonance (EPR) spectra of spin-labeled samples of ribonuclease A were recorded on a Brüker 414S spectrometer operating at 9.5 GHz and equipped with a rectangular cavity. Samples were dissolved in 0.2 M sodium acetate buffer (pH 5.48) and held in an aqueous flat cell (Scanlon Co.). Temperatures were monitored with a Cu-constantan thermocouple inserted to a point just above the flat part of the cell and external to the microwave cavity. The EPR spectra of digestion fragments (see below) of spin-labeled ribonuclease A were recorded on a Varian V4595 spectrometer. The same buffer and cell geometry were used, and the microwave power was kept below 5 mW on both spectrometers.

CNBr-digestion of HSL-ribonuclease A was performed by the method of Gross and Witkop (7). Peptide fragment C1 (residues 1-13) was separated from the remainder of the protein on a 2.3 x 162 cm column of Sephadex G-25 in 0.2 N acetic acid. Digestion of carboxymethylated reduced BSL-ribonuclease A with trypsin was performed as described by Hirs et al. (8). The fragment comprising residues 105-124 was isolated on a Dowex 50W-X2 column (1.9 x 5.0 cm) equilibrated with 2 M ammonium formate buffer (pH 5.1) by the general technique of Hirs et al. (8)

Amino acid analyses were performed on a Technicon TSM Automatic Analyzer, following hydrolyses in 6 N HCl at 105°C for 24 hrs.

Enzymatic activity was determined by the method of Crook et al. (9) using cytidine-2':3'-cyclic phosphate as substrate.

RESULTS

The reaction of BSL with ribonuclease A has been described previously (6,10). The careful investigation of Daniel et al. (6) characterized the major product as ribonuclease A spin-labeled at the 3-nitrogen of His-105. We repeated their characterization procedure (with the exception of the Edman degradation conducted on the tryptic fragment (105-124), and confirmed all of their observations. The chromatographic pattern of the desalted BSL-ribonuclease A reaction mixture is shown in Fig. 1a. The material from peak I has a specific activity of 88% ± 5% of native ribonuclease A; that of peak II is 93% ± 5%. The last peak (III) is small, rather broad and, after being pooled, lyophilized and redissolved in sodium acetate buffer (100 µl), gave rise to a barely detectable EPR signal. Daniel et al. (6) were able to obtain considerably more of this labile side product (which they identified as ribonuclease A spin-labeled at a Met residue). This minor difference between our results and those of Daniel

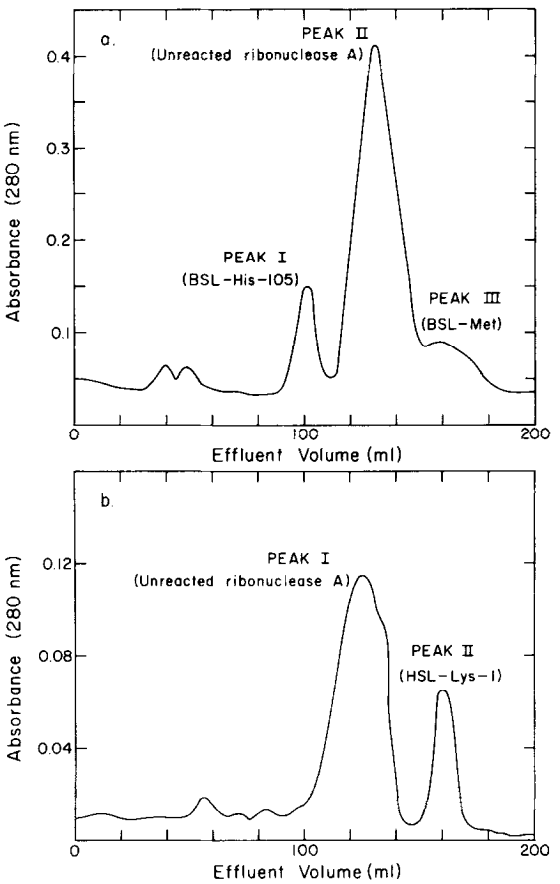

Figure 1. Chromatographic patterns for the desalted reaction mixtures on BioRex 70 (-400 mesh). (a) BSL-ribonuclease A, (b) HSL-ribonuclease A.

et al. (6) is probably due to the longer reaction time used in this work (110 hrs instead of 72 hrs). It, thus, seems well established that the species BSL-ribonuclease A (peak I) consists of the enzyme with a single spin label attached at His-105.

The reaction mixture of HSL with ribonuclease A produced the chromatographic pattern shown in Fig. 1b. Peak I eluted where unreacted ribonuclease was found to elute and, although a very weak EPR signal could be obtained, it was mostly unreacted material. Its specific activity is 92% ± 5% of native ribonuclease A. Peak II, however, contains the bulk of the spin-labeled mate-

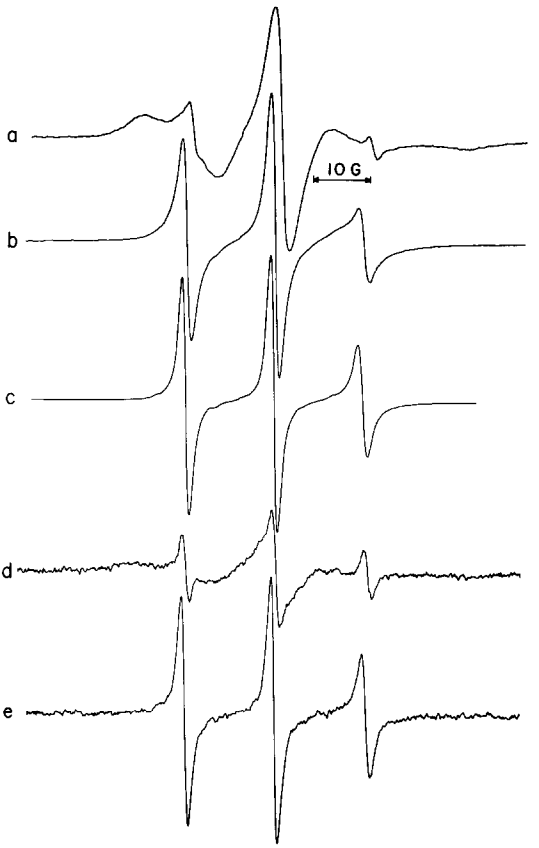

Figure 2. EPR spectra of spin labeled ribonuclease A derivatives as a function of temperature in 0.2 M acetate buffer, pH 5.5. (a) BSL-His-105, 8°C, (b) BSL-His-105, 55°C, (c) BSL-His-105, 65°C, (d) HSL-Lys-1, 3°C, (e) HSL-Lys-1, 65°C.

rial, with a specific activity of 89% ± 5%. When this product was digested with CNBr, all of the spin-labeled material was found in the C1 fragment. This fragment was unaffected by aminopeptidase digestion. The spin label HSL has been used to probe tRNA molecules (2,3,11), and is a specific acylating reagent for α-amino groups. The product of acylation is acid sensitive (12), and consequently the residue that bears the spin label cannot be determined by the normal acid hydrolysis procedure used for amino acid analysis. However, the known specificity of HSL, the location of the spin label in the C1 fragment of ribonuclease A, and the resistance of this tridecapeptide to the action of

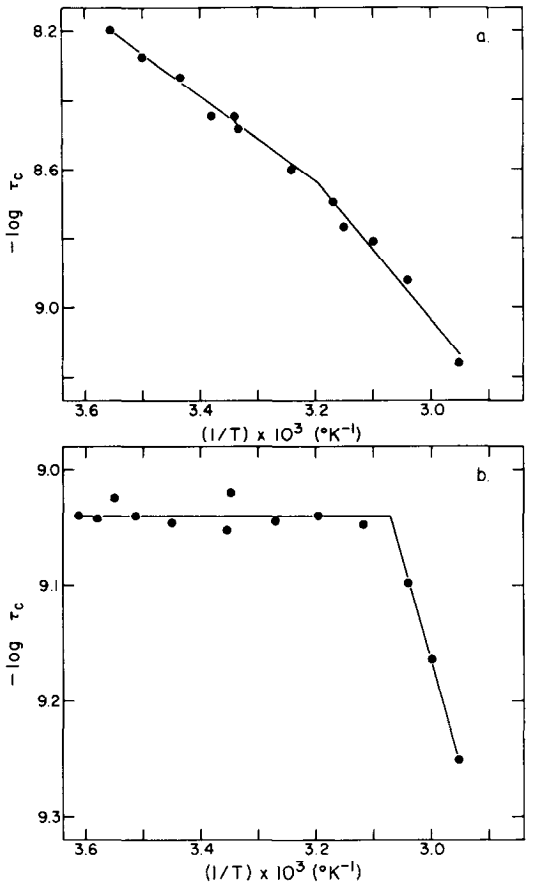

Figure 3. Arrhenius plots of spin labeled ribonuclease derivatives, with τ_c in seconds. (a) mobile component of BSL-His-105, (b) HSL-Lys-1.

aminopeptidase strongly suggests that the α-amino group of Lys-1 bears the spin label.

The EPR spectra of the two spin-labeled derivatives BSL-His-105 and HSL-Lys-1 were recorded over the temperature range 3°-65°C. Representative spectra are shown in Fig. 2. It is within this temperature range that the thermal transitions of ribonuclease A at pH 5.5 are known to occur (13). This pH was chosen to facilitate comparison with the results of Smith (10). Spectra recorded at the same temperature before and after heating to 64°C were found to be superimposable as long as heating above 60°C was not prolonged for more

than about 30 min; thus, the process of unfolding is reversible. The quadratic rotational correlation time of the spin label was calculated at each temperature using the equation (14):

$$\tau_c \text{ (nsec)} = (0.606)\Delta H_0[\sqrt{h_0/h_{+1}} + \sqrt{h_0/h_{-1}} - 2] \quad (1)$$

Here h_0, h_{+1}, h_{-1} are the amplitudes of the center, low field and high field lines, respectively, and ΔH_0 is the width of the center line in gauss. The equation is valid only if the motion of the spin label is isotropic (14). The results are presented in Fig. 3 as Arrhenius plots. The lines drawn in this Figure are least squares fitted lines, and it is clear that there exists a single sharp break point about 40°C and 53°C, respectively.

DISCUSSION

The EPR spectra of BSL-His-105 ribonuclease A at low temperatures consist of a mobile (sharp) and relatively immobilized (broad) component. The broad component could be detected until 55°C (cf. Fig. 2b and 2c), while the sharp component acquires increased mobility at 40° ± 5°C (see Fig. 3a). Dugas and Gaudet (15) have observed an analogous spectrum (two components, one mobile and one less mobile) with a spin label attached to the active site of staphylococcal protease, and have speculated that it can be produced by a single label in two different orientations. Such an interpretation seems possible in this case as well, with the mobile orientation becoming more mobile at 40°C very likely because of freer side-chain rotations, and the more restricted orientation persisting until 55°C. Since the backbone structure around His-105 is not believed to change until 50°-60°C (13), our interpretation is therefore consistent with the idea of a local increase in the motional freedom of the spin label around 40°, followed at 55° by a more complete loosening of the peptide chains surrounding His-105. This situation is quite similar to that which exists for Tyr-92 in ribonuclease A, where the side chain acquires conformational freedom above 25°C but the backbone in this region retains its native conformation even at 60°C (13).

The spectra of HSL-Lys-1, however, display a markedly different temperature dependence. For example, the spectrum at low temperature (see Fig. 2d) shows only a small fraction of the spin label in a strongly immobilized orientation. Consequently, the shape of the EPR signal indicates that the label is not buried in any way by the protein backbone, compared to the behavior encountered with BSL-His-105, but is rather oriented towards the solvent and rotates quite freely. More important is the fact that almost no change in τ_c is observed until 53° ± 3°C. This experimental observation correlates very well with the hypothesis that the N-terminal portion of ribonuclease A is not affected by temperature until 55°-65°C, for the reason cited in ref. 13.

These results clearly demonstrate that the thermally induced unfolding of ribonuclease A is sequential (13) and that the N-terminal α-helix tail of the protein unfolds at a late stage (13). This appears to be at variance with the conclusion of Roberts and Benz (16,17), based on NMR evidence.

Since the curves in Fig. 3 are Arrhenius plots ($\log \tau_c$ = const. - ΔH_{spin}/ 2.3 RT), their slopes provide a measure of the spin enthalpy of each species (11). For BSL-His-105, ΔH_{spin} = 5.3 ± 0.05 and 9.6 ± 0.9 kcal/mole below and above the break point (40° ± 5°C), respectively. These are in reasonable agreement with those of Smith (10). For HSL-Lys-1, ΔH_{spin} = 0.0 ± 0.2 below 53° ± 3°C, and 4.2 ± 0.2 kcal/mole above this temperature.

Conclusions. The results reported here demonstrate the potential of EPR spectroscopy for studying protein unfolding. By introducing a spin probe at a specific residue, it is possible to monitor the conformational integrity of the protein in the neighborhood of that residue. By using probes of differing specificity, it is possible to order the steps which occur along the pathway(s) of protein unfolding, provided that the spin labels do not perturb its three-dimensional structure significantly.

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