Spin-labeled polyribonucleotides

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ABSTRACT

Poly (U), poly (C) and poly (A) were spin labeled with N-(2,2,5,5-tetramethyl-3-carboxypyrroline-1-oxyl)-imidazole. This spin label interacts selectively with 2' OH ribose groups of polynucleotides and does not modify the nucleic acid bases. The extent of spin labeling is not dependent upon the nature of the base and is entirely determined by rigidity of the secondary structure of the polynucleotide. The extent of modification for poly (U), poly (C) and poly (A) was 4.2, 1.7 and 1.5 per cent, respectively, the secondary structure of the polynucleotides being practically unchanged. Some physico-chemical properties of the spin-labeled polynucleotides were investigated by ESR spectroscopy. Rotational correlation times of the spin label and activation energy of its motion were calculated.

INTRODUCTION

The spin labeling technique has been widely used to study the structural organization and conformational mobility of biological macromolecules and their complexes (1,2). One of the essential problems which arise in using this technique is selective introduction of a spin label (nitroxyl radical) by means of covalent or non-covalent bond into a certain site of the object. Exact localization of the spin label is necessary for correct interpretation of experimental results obtained by ESR spectroscopy. The problem of selective modification of certain sites of macromolecule by spin labels solved rather easily for proteins and enzymes, offers certain difficulties in the case of nucleic acids. In the literature, there are only data on selective spin labeling tRNA's (3) which contain a minor component whose chemical reactivity is significantly different from that of other nucleotide residues.

All labels used up to now for spin labeling of polynucleotides (4-8), tRNA's (9-15) and DNA's (16-17) are constructed on the basis of chemical agents alkylating or acylating the nucleic bases and are, as a rule, not highly specific. No attempts, however, were made to introduce the spin label into the ribose-phosphate backbone of the polynucleotide chain, although the chemical modification of 2' OH ribose groups can be undoubtedly carried out with high selectivity.

This study concerns the chemical reactivity of the nitroxyl radical N-(2,2,5,5-tetramethyl-3-carboxylpyrroline-1-oxyl)-imidazole with synthetic polyribonucleotides. The label, suggested initially for spin labeling of tyrosine residues in proteins (18) interacts highly specifically with 2'(3') OH ribose groups of nucleosides and nucleotides and does not modify the nucleic bases (19). Poly (U), poly (C) and poly (A) were spin-labeled and the dependence of rotational mobility of the spin label, bound covalently with 2' OH ribose groups, on the conformation of these polynucleotides in the solution was studied by ESR spectroscopy.

MATERIALS AND METHODS

Spin Label

The spin label N-(2,2,5,5-tetramethyl-3-carboxylpyrroline-1-oxyl)-imidazole was synthesized from 2,2,5,5-tetramethylpyrroline-1-oxyl-3-carboxylic acid and carbonyldiimidazole according to the procedure suggested by Barrat et al. (18). The label's purity was controlled by IR-spectra and elementary analysis (carbon, hydrogen, nitrogen). The polynucleotides, poly (U), poly (C) and poly (A) from "Reanal" firm (Hungary) were thoroughly dialyzed in the cold, first against 0.1 M EDTA, then against twice distilled water until the UV-absorption in the dialysate was completely eliminated. The dialysed preparations were lyophilized and stored at -5°C.

Preparation of Spin-Labeled Polyribonucleotides

0.2 mmol of spin label were dissolved in 0.4 ml of absolute tetrahydrofuran and added to the solution containing 0.3 mmol of polynucleotide dissolved in 2.6 ml of 0.15 M Na-cacodylate

buffer, pH 7.5. The reaction mixture was incubated at room temperature for 5 hours. At the end of the reaction the spin-labeled polymers were isolated, free of unreacted spin label, in the following way: ethanol was added to the reaction mixture up to 85% concentration, the mixture was cooled to 0°C and centrifuged at 9000 r.p.m. for 30 min. The supernatant was removed by decantation and the precipitate was dissolved in water and then exhaustively dialyzed against 0.1 M EDTA and finally, against twice distilled water. An aliquot of the final dialysis bath was monitored by ESR to establish the absence of detectable amounts of unreacted spin label. The covalent nature of the spin label - polynucleotide linkage was indicated by gel filtration on Sephadex G-50 of spin labeled polynucleotides after incubation at 80°C for 30 min. The elution profile did not show any peak corresponding to the unbound label. The extent of modification of the polynucleotides by the spin label was determined by comparing the ESR spectrum of free label and that of spin labeled polynucleotide after hydrolysis in 3 N KOH for 24 hours.

Measurements of UV-Spectra

UV-spectra of the spin-labeled and non-labeled polynucleotides were recorded at room temperature on a Specord UV-VIS (Zeiss, Jena) spectrophotometer. The UV melting temperature profiles were measured on a VSU-2P spectrophotometer, equipped with a thermostated cell holder. The concentration of polynucleotide solutions was estimated from optical absorbances using the following analytical wavelengths and extinction coefficients: poly (U) - 260 nm, $9.2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$, poly (C) - 267 nm, $6.6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$, poly (A) - 257 nm, $10.5 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (20).

ESR-Measurements

ESR-spectra were measured on a RE 1306 (USSR) spectrometer using a microwave power within 10 mW to avoid saturation. The samples containing spin labeled polynucleotides were placed into glass capillaries of about 0.5 mm inner diameter. The rotational correlation time, τ_c , of the spin label was calculated from the following expression:

$$\tau_c = 6.5 \times 10^{-10} \times \Delta H_0 \times (\sqrt{h_0/h_+} + \sqrt{h_0/h_-} - 2) \text{ sec}$$

where ΔH_0 is peak-to-peak separation of the central hyperfine component of the ESR-spectrum in gauss, h_0 , h_+ and h_- are the amplitudes of the center, low field and high field lines, respectively. In the cases when ESR-spectra represent a complex superposition of ESR-spectra of spin labels with different immobilization degree, the h_0/h_+ ratio was taken as a parameter to characterize the label mobility.

All ESR-spectra were recorded three times with scan time of 8 min, and the mean value from three measurements was used in the calculations.

Temperature measurements were performed with a thermostatic unit by passing a flow of dry nitrogen at fixed temperature through a Dewar quartz tube placed into a microwave cavity. The temperature was measured with an accuracy of $\pm 0.5^\circ\text{C}$ at the outlet of the microwave cavity using a copper-constantan thermocouple. Samples were allowed to equilibrate for 10 minutes before measurements.

RESULTS AND DISCUSSION

The spin label N-(2,2,5,5-tetramethyl-3-carboxypyrroline-1-oxyl)-imidazole reacts with poly (U), poly (C) and poly (A) under rather mild conditions. The chemical structure of the nucleotide residue in the polymer after acylation of the 2' OH ribose group with the spin label is presented in Figure 1.

Figure 1. Chemical structure of the polymnucleotide chain fragment with the spin label attached.

The extent of spin labeling of polynucleotides depends on the ratio between the initial concentrations of the reacting components and on the time of incubation of the reaction mixture. Under similar conditions the extent of spin labeling is determined by the secondary structure of the polynucleotide. It can be seen from Table 1 which gives the percentage of the spin labeled residues in poly (U), poly (C) and poly (A) under the conditions described in METHODS section.

A distinct correlation between the secondary structure of the polynucleotides and the extent of labeling is observed. Poly (C) and poly (A) which have rather rigid single-helical structures in solution at neutral pH (21,22), bind the label to a significantly lesser degree than does poly (U) which has no ordered structure at room temperature (23).

In spite of the higher modification degree as compared to the labeling of polynucleotides by spin labels alkylating or acylating the nucleic acid bases (4,5,15), the acylation of 2'OH ribose groups by N-(2,2,5,5-tetramethyl-3-carboxypyrroline-1-oxyl)-imidazole does not change essentially polynucleotide conformation in the solution. It is confirmed by the fact that UV- and CD-spectra of the spin-labeled and non-modified polynucleotides practically coincide, the value of hyperchromism and the UV melting temperature profiles of poly (C) and poly (A) at acidic pH values does not change either.

Figure 2 presents ESR-spectra of the spin labeled poly (U), poly (C) and poly (A) under the conditions when these polynucleotides form single-stranded structures, and Table 2 shows the

Table 1. Extent of the chemical labeling of polynucleotides by the spin label N-(2,2,5,5-tetramethyl-3-carboxypyrroline-1-oxyl)-imidazole

Spin-labeled polynucleotide	The percentage of spin-labeled residues in the polynucleotide
poly (U)	4.2 $\pm$ 0.2
poly (C)	1.7 $\pm$ 0.2
poly (A)	1.5 $\pm$ 0.2

Figure 2. ESR-spectra of the spin-labeled polyribonucleotides in 10 mM NaCl, 10 mM tris-HCl, pH 7.5, of: a - poly (U) conc. 3.87 mM; b - poly (C), conc. 5.07 mM; c - poly (A), conc. 5.38 mM. ΔH_0 is the peak-to-peak separation of the central hyperfine component in gauss; h_0 , h_+ and h_- are the amplitudes of the central, low field and high field lines, respectively.

rotational correlation times of the spin label, τ_c .

It is seen that in the rigid single-stranded structures of poly (A) and poly (C) the spin label is significantly less mobile than in the disordered structure of poly (U).

Table 2. Rotational correlation times, τ_c , of spin-labeled polyribonucleotides in 10 mM NaCl, 10 mM tris-HCl, pH 7.5, 21°C

Spin-labeled polynucleotide	τ_c , sec.
poly (U)	1.24×10^{-9}
poly (C)	2.53×10^{-9}
poly (A)	3.21×10^{-9}

Base-Acid Titration of Spin-Labeled Polymucleotides

During transition from neutral to acidic pH poly (C) and poly (A) form protonated highly ordered double-helical structures (24,25), and in the case of spin-labeled polymucleotides this manifests itself in abrupt changes of the ESR-spectra. Figure 3 presents ESR-spectra of the spin-labeled poly (C) and poly (A) in 10^{-2} M NaCl at pH 4.0 which are a superposition of ESR-spectra of at least two types of spin labels with different degree of immobilization.

The first type of the signal corresponds, evidently, to the highly immobilized spin labels ($\tau_c \gg 10^{-9}$ sec) located in the double-helical areas of polymucleotides, while the second one arises from the rapidly rotating labels ($\tau_c < 10^{-9}$ sec.) attached to the end nucleotide residues. The comparison of the ESR-spectra of spin labeled polymucleotides shows that the protonated double-helical poly (A) has a more rigid structure than the semiprotonated poly (C). The ratio between the amplitudes of the center and low field lines changes abruptly during titration, and we have taken the h_0/h_+ ratio as a parameter describing

Figure 3. ESR-spectra of the spin-labeled polymucleotides in 10 mM NaCl, 10 mM Na-acetate, pH 4.0, of: a - poly (C), b - poly (A).

the changes in ESR-spectra of the spin-labeled polynucleotides. The pH-dependence of this parameter during titration of the spin-labeled poly (U), poly (C) and poly (A) is shown in Figure 4. It is seen that at pH 5.8 there is a sharp cooperative transition of the spin-labeled poly (C) and poly (A) into double-helical structures, while the ESR-spectrum of the spin-labeled poly (U) does not depend on pH of the medium.

These data agree with the results of potentiometric and UV-spectrophotometric titrations of these polynucleotides: pK 5.7 for poly (C) and 5.9 for poly (A) (24,27). A slight decrease of h_0/h_+ at pH close to pK confirms the existence of the flexible poly (C) structure observed earlier by Guschlbauer (26). Probably, some intermediate flexible structure is formed also at protonation-induced transition of poly (A) from the single-stranded to the double-helical structure.

Temperature Dependence of ESR-Spectra of the Spin-Labeled Polynucleotides

Figure 5 shows the temperature dependence of rotational correlation times of the spin labels attached to poly (U), po-

Figure 4. Dependence of h_0/h_+ on pH for: (o) poly (U), (Δ) poly (C) and ($\bullet$) poly (A) in 10 mM NaCl at 21°C.

Figure 5. Dependence of the spin label correlation time, τ_c , on absolute temperature for spin labeled: (●) poly (U), (▲) poly (C) and poly (A) (○) in 10 mM NaCl, 10 mM tris-HCl, pH 7.5.

ly (C) and poly (A). No discontinuities reflecting a sudden change in the polynucleotide structures were observed in ESR temperature profiles. This is consistent with the well-known non-cooperative character of melting these single-stranded polynucleotides as revealed by UV and CD studies.

Activation energies of spin label motion in the polynucleotide structures can be obtained from the dependences of τ_c on the absolute temperature, according to the Arrhenius equation:

$$\tau_c = \tau_0 \cdot \exp (-\Delta E/RT)$$

where ΔE is activation energy. The ΔE values are given in Table 3.

It seems tempting to relate these values to the ΔH of uns-

Table 3. Activation energies (ΔE) of spin label motion in single-stranded polynucleotide structures. Conditions are the same as in Figure 5.

Spin-labeled polynucleotide	ΔE , kcal/mole
poly (A)	7.5 ± 0.2
poly (C)	6.9 ± 0.2
poly (U)	5.7 ± 0.2

taking in polynucleotides. However, it should be remembered, that the ΔE observed are a measure of energetic barrier for the spin label motion and are determined mainly by change with temperature in nearest surrounding for the spin label. The near ΔE values (Table 3) obtained with different polynucleotides show, probably, the similar surrounding for the spin label. Thus, it seems most probable, that the spin label attached at 2' position of ribose groups is sensitive, in the first place, to changes in ribose-phosphate backbone of polynucleotides. Undoubtedly, definite contribution to the ΔE is made also by stacking interaction between nucleic acid bases. The differences between ΔE for poly (U), poly (C) and poly (A) clearly show it. However, there is no direct relationship between activation energy for the spin label motion, ΔE , and enthalpy of unstacking in polynucleotides, ΔH .

Analysis of the data presented in Tables 1,2 and 3 reveals a distinct correlation between the degree of modification of the polynucleotides by N-(2,2,5,5-tetramethyl-3-carboxypyrroline-1-oxyl)-imidazole, rotational correlation time of the spin label and activation energy of its motion. For example, poly (U) which has no ordered structure at room temperature shows a better reactivity with spin label than other polynucleotides, the rotational mobility of the label is less restricted and the activation energy of its motion is lower.

As it has been already mentioned, at acidic pH the h_o/h_t ratio can serve as a parameter characterizing the conformational state of the spin-labeled poly (C) and poly (A). The temperature dependence of this parameter for spin-labeled poly (A) at pH 4.0 is presented in Figure 6. The temperature dependence

Figure 6. Dependence of h_0/h_+ on temperature for the spin labeled poly (A) in 10 mM NaCl at: (o) pH 7.5, 10 mM tris-HCl and (•) pH 4.0, 10 mM Na - acetate.

of h_0/h_+ for the spin-labeled poly (A) in the single-helical conformation is also shown there for comparison.

It can be seen that at pH 4.0 the temperature dependence of h_0/h_+ is S-shaped, thus, demonstrating the cooperative melting of the protonated double-helical poly (A). For the single-helical poly (A) the exponential decrease of h_0/h_+ is observed.

The h_0/h_+ - temperature profile is very broad for the spin-labeled poly (A) at pH 4.0 and show, at first sight only, a poor cooperativity of melting. However, the reason of such broad ESR melting of poly (A) is quite another and is related to one specific peculiarity of spin labeling, that distinguishes this method sufficiently among most spectral techniques. This peculiarity is conditioned by a very strong dependence of spin label correlation time (as well as of the parameter h_0/h_+ , used in this study) upon temperature, even in the absence of any structural transitions. Thus, the dependence of h_0/h_+ upon temperature for the protonated spin-labeled poly (A) (Figure 6) has a complex nature and is not only the consequence of poly (A) melting. For this reason, no conclusion can be made about the coope-

rativity of temperature-induced structural transitions from the ESR data only.

Indeed, the spin-labeled poly (A) reveals high cooperativity in the acid titration (Figure 4). Moreover, UV temperature melting of protonated spin-labeled poly (A) takes place within $9\pm 2^\circ\text{C}$ and does not differ, practically, from UV melting of the unmodified polynucleotide. UV melting temperatures were found to be 76°C and 78°C for spin-labeled and non-labeled poly (A), respectively. These values are close to those obtained elsewhere (28) and differ sufficiently from ESR melting temperature, 50°C (Figure 6).

Thus, the ESR melting temperature (designated often as T_m^{SP}) is some "apparent" value and, in principle, does not mean the middle of transition. But now we cannot say, how T_m^{SP} is related to the T_m obtained by other spectral methods.

In contrast to the above conclusions, the covalent introduction of spin labels into polynucleotide structure can result in "real" lowering of T_m due to some destabilization of polynucleotide structure in the sites of spin label attachment. As follows from this study, the chemical modification of the small part of ribose residues by the spin label does not result in a drastic change of polynucleotide structure.

The Covalent Nature and Stability of the Spin Label - Polynucleotide Linkage

The covalent nature of spin label - polynucleotide linkage was confirmed in the following way: the samples of spin-labeled polymers were incubated at 80°C for 30 min. and subjected to gel filtration on Sephadex G-50 after cooling. The absence in the elution profile of the peak corresponding to the free spin label suggests that such treatment does not result in release of spin label from the polynucleotides, which would be impossible in the case the bonds were non-covalent. The covalent nature of the linkage is indicated also by the inability to remove the spin labels from the polynucleotides by exhaustive dialysis against twice distilled water.

The bond of spin label with polynucleotides is stable in the range of pH 3-9 and temperatures $0-80^\circ\text{C}$. With these pH and temperatures, there are no noticeable changes in ESR-spectra of

the spin labeled polynucleotides during two hours.

CONCLUSION

The spin label N-(2,2,5,5-tetramethyl-3-carboxypyrroline-1-oxyl)-imidazole interacts covalently and selectively with 2' OH ribose groups of polyribonucleotides. However, its application can be much wider. Our preliminary experiments have shown that this label can be used for spin labeling of tRNA's, DNA's of T-even phages containing the glucosylic cytidine residues and calf thymus DNA. In the latter case the label is bound, probably, with the terminal OH-groups, as well as with OH-groups at the sites of single DNA breaks. As it has been already shown (19), the label does not interact with nucleic bases and modifies selectively the OH ribose groups. Such introduction of the spin label into polynucleotides does not exclude the possibility of hydrogen bonding and the realization of other interactions between the nucleic bases. The label attached to the 2' OH ribose groups of the polynucleotides is, as it follows from this study, a sensitive probe responding to changes of their conformation in the solution.

In our next communication (29) it is shown that such spin labeled polynucleotides can be used for obtaining valuable information about intermolecular interactions in the polynucleotide - monomer systems that is difficult and even impossible to gain by other physico-chemical methods.

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