The synthesis of poly-4-thiouridylic acid and its effect on protein synthesis in a cell free system derived from rat liver

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ABSTRACT

The synthesis of poly-4-thiouridylic acid by thiolation of polycytidylic acid is described. A quantitative thiolation was achieved without any cleavage of the phosphodiester bond. The inhibitory effect of the poly-4-thiouridylic acid on protein synthesis in a cell free system derived from rat liver was investigated.

INTRODUCTION

The use of oligo- and polynucleotides as synthetic mRNA has become a useful tool in the investigation of protein synthesis in vitro¹. Modified polynucleotides however can be used in order to study some specific aspects involved in the complex mechanism of protein synthesis.

Several attempts to introduce 4-thiouracil, a sulfur analog of uracil, into oligo- and polynucleotides were reported. Simuth et al.² synthesized enzymatically polynucleotides containing 4-thiouridine, by the polymerization of 4-thiouridine-5'-diphosphate with polynucleotide phosphorylase. The 4-thiouridine-5'-diphosphate is a poor substrate for polynucleotide phosphorylase and therefore, the yield of the polymerization product is low. In order to obtain a copolymer of cytidylic- and 4-thiouridylic acid, Favre et al.³ thiolated polycytidylic acid with H₂S. 36% thiolation was achieved when mild conditions of amino-thiol exchange reaction reported by Miura et al.⁴ was used. Recently, Vormbrock et al.⁵

synthesized enzymatically oligomers containing 4-thiouridylic acid and tested their codon-anticodon interaction in a ribosomal nonenzymatic binding system derived from E. coli.

In the present communication the synthesis of poly-4-thiouridylic acid by complete thiolation of polycytidylic acid is described. The inhibitory effect of the synthetic poly-4-thiouridylic acid on a protein synthesizing system derived from rat liver was investigated.

MATERIALS AND METHODS

[¹⁴C]phenylalanine, specific activity of 522 mCi/mmole was purchased from Amersham England, Polycytidylic acid (S₂₀ value of 5.4) was prepared by the polymerization cytidine-5'-diphosphate with polynucleotide phosphorylase from *M. lysodeikticus*. Pancreatic RNase was purchased from Sigma, U.S.A.

Preparation of poly-4-thiouridylic acid. Polycytidylic acid (300 mg) was dissolved in 6 ml of pyridine-water mixture 1:5 (v/v) and cooled to -70° C in a 50 ml stainless steel cylinder. A solution containing 10 ml liquid H₂S and 5 ml pyridine was added. The container was sealed and heated to 37° C for 12 days. The content was evaporated to dryness and the residue obtained was suspended in 1 ml of water and centrifuged. Two volumes of ethanol and 0.1 M NaCl (final concentration) were added to the supernatant and the poly-4-thiouridylic acid which precipitated out was isolated by centrifugation. The polymer was passed through a G-25 Sephadex Column (1 x 40 cm) and eluted with 0.1 mM mercaptoethanol. Light was avoided throughout the whole procedure to prevent photoreactions.

Enzymatic hydrolysis. 5.0 A₃₃₀ units of poly-4-thiouridylic acid in 25 µl of 0.1 M phosphate buffer, pH 7.0, were incubated overnight with 10 µg pancreatic RNase, at 37°C. The hydrolysis products were separated by thin layer chromatography on aluminium cards coated with cellulose powder (Riedel de Haen, Hannover, G. FR). The solvent system used was saturated ammonium sulphate, 1 M sodium acetate, isopropanol (80:18:2). The separated hydrolysis products were extracted with water and their UV absorption was measured.

Absorption spectra were recorded on a Cary 14 spectrophotometer, CD spectra were recorded on a Cary 60 spectropolarimeter equipped with 6001 CD attachment. The extinction coefficient at 330 nm was taken as 18000 per residue, according to Simuth et al.²

Sedimentation coefficient of the poly-4-thiouridylic acid was determined by the Beckman model E analytical ultracentrifuge using Schlieren optics. The poly-4-thiouridylic acid was dissolved in 10 mM phosphate buffer pH 7.0 containing 0.1 M NaCl to a concentration of 6 mg/ml. The run was performed at 23°C and the S value was corrected to 20°C.

The conversion of poly-4-thiouridylic acid to poly uridylic acid was performed according to Scheit⁶.

Polyribosomes from rat liver were prepared according to Bloemendal et al.⁷ and the incorporation of phenylalanine into protein and into polyphenylalanine was measured. The incubation mixture (37°C) contained in final volume of 220 µl the following: Tris HCl pH = 7.4, 60 mM, KCl 50 mM; MgCl₂ 10 mM; DTT 1 mM; GTP 0.5 mM; ATP

0.5 mM; PEP 5 mM; [^{14}C]phenylalanine 0.2 mCi; mixture of 19 amino acids 50 μM each: PEP kinase (Boehringer) 10 μg ; tRNA (rat) 20 μg ; polyribosomes 1.0 A_{260} (if not stated otherwise), and aliquots of the enzymatic fraction (necessary for optimal incorporation) and polyuridylic acid (when added) 40 μg . At indicated time intervals samples (50 μl) were withdrawn, washed and processed according to Bollum⁸. Samples were counted for 5% hot TCA insoluble material in a liquid scintillation counter. Amino acylation of tRNA was measured according to Hochberg *et al.*⁹

RESULTS AND DISCUSSION

Muira *et al.*⁴ reported on the amino thiol exchange reaction in cytidine containing dinucleoside monophosphate. A modified procedure for the thiolation of polycytidylic acid resulted in the formation of poly-4-thiouridylic acid. When the thiolated polycytidylic acid was hydrolyzed by pancreatic RNase, 4-thiouridine-3'-phosphate was the sole product. No cytidine -3'-phosphate or uridine-3'-phosphate was found. In an experiment where the thiolation of polycytidylic acid was performed for a shorter period of time (3 days) the extent of the thiolation was 80%.

The poly-4-thiouridylic acid sedimented as a sharp peak corresponding to a S_{20} value of 2.5. This value is smaller than that of the starting material polycytidylic acid (5.4). However, no oligonucleotides were found during the purification steps. Polycytidylic acid is known to have a compact conformation in solution. The change in the S value as a result of thiolation can be explained by less compact conformation of poly-4-thiouridylic acid. This is in agreement with

Favre et al.³ who found that already 2% thiolation of polycytidylic acid reduced the S value from 13 to 6.6. Further thiolation to the extent of 36% reduced the S value to 6. It can be concluded therefore, that the drastic conditions used for quantitative thiolation of polycytidylic acid did not cause any cleavage of the phosphodiester bond.

Three spectra of the poly-4-thiouridylic acid are shown in Fig. 1. For comparison, the spectra of the monomer-4-thiouridine-2'(3')monophosphate and the dimer-4-thiouridylyl-(3'-5')-4 -thiouridine are shown. The spectra of the three compounds do not differ in the short UV region (below 280 nm). The long UV absorption (around 330 nm) is significantly changed in the dimer and the polymer (Fig. 1A). Hyperchromicity upon hydrolysis is seen at 335 nm (Fig. 1B), 4% for the dimer and 19% for the polymer. A small hypochromicity can be seen at 358 nm for the dimer and at 375 for the polymer. CD spectra (Fig. 1C) show that the dimerization of 4-thiouridylic acid causes a splitting of the single asymmetric transition of the monomer at 335 nm into two transitions with opposite signs; a positive cotton effect at 348 nm and a negative one at about 315 nm. Polymerization of 4-thiouridylic acid gives the same picture but with about 3 nm blue shift in comparison to the dimer. This shift is less pronounced in the absorption spectrum (Fig. 1A).

Fig. 1. Absorption spectra of poly-4-thiouridylic acid. A. Absorption spectrum; B. Changes in absorbance caused by enzymatic hydrolysis by pancreatic RNase; C. Circular dichroism. The spectra of 0.5×10^{-4} M (residue) of (● - ●) 4-thiouridine 2'(3')phosphate (x - x) 4-thiouridylyl-3'-5'-4-thiouridine (Δ - Δ) poly-4-thiouridylic acid. All spectra were recorded as described in methods (solvent 10 mM phosphate buffer pH = 7.0 containing 0.1 M NaCl).

Fig. 2. The effect of poly-4-thiouridylic acid on the endogeneous and poly U directed [^{14}C]phenylalanine incorporation. A. Endogeneous incorporation; B. Poly U directed incorporation; For experimental details see text. (● - ●) No addition; (x - x) 0.2 μg ; (▲ - ▲) 1.0 μg ; (■ - ■) 4.0 μg ; (○ - ○) 20 μg ; (□ - □) 40-80 μg of poly-4-thiouridylic acid.

The effect of poly-4-thiouridylic acid on the incorporation of [^{14}C] phenylalanine into 5% hot TCA insoluble material is shown in Fig. 2A. This incorporation is endogenous mRNA dependent. 0.05 μg - 4 μg of poly-4-thiouridylic acid did not effect the incorporation. 20 μg of poly-4-thiouridylic acid however caused about 40% inhibition and the addition of 40-80 μg of poly-4-thiouridylic acid caused a complete inhibition.

A different dose-response curve is obtained when poly U dependent polyphenylalanine formation is measured (Fig. 2B). 0.05 μg and 0.1 μg of poly-4-thiouridylic acid did not inhibit the incorporation of [^{14}C]phenylalanine. 1.0 μg already caused 50% inhibition and 4 μg of poly-4-thiouridylic acid caused a complete inhibition of the poly U dependent [^{14}C]phenylalanine incorporation. Similar results were obtained when a copolymer of cytidylic acid and 4-thiouridylic acid 1:4 was used as an inhibitor in the above described incorporation systems. At concentrations of poly-4-thiouridylic acid above 4 μg both the endogeneous mRNA and the poly U dependent incorporation of [^{14}C]phenylalanine is inhibited, whereas at concentrations below 4 μg , only the poly U directed polyphenylalanine synthesis is inhibited. Polycytidylic acid (40 μg) did not inhibit the [^{14}C]phenylalanine incorporation. In order to elucidate whether monomers or dimers of 4-thiouridylic acid can cause the same inhibition effect, the effect of 40 μg of 4-thiouridine; 4-thiouridine-2'(3')-phosphate; 4-thiouridine-5'-diphosphate and 4-thiouridylyl-3'-5'-4-thiouridine on the endogeneous and poly U dependent incorporation was examined. No measurable inhibition was found with any of these compounds. (Results not shown). Poly U derived from the oxidation of poly-4-thiouridylic acid promoted the incorporation of

phenylalanine to polyphenylalanine to the same extent as the control value.

In order to elucidate whether the inhibition of phenylalanine incorporation by high concentrations of poly-4-thiouridylic acid is caused by inhibition of aminoacylation of the tRNA the following experiment was performed: 40 μ g of poly-4-thiouridylic acid were added to an aminoacylation assay system. The formation of phenylalanyl- or valyl-tRNA was not inhibited. However, the same amount of poly-4-thiouridylic acid completely inhibited the poly U directed [14 C]phenylalanyl-tRNA binding to 40S + 60S subunits (results to be published).

Table I: The inhibition of phenylalanine incorporation into polyphenylalanine by poly-4-thiouridylic acid as a function of polyribosomes concentration.

Polyribosomes A ₂₆₀ units	% inhibition	Polyribosomes inactivated A ₂₆₀ units
0.5	90	0.45
1.0	36	0.36
2.0	23	0.46
3.0	11	0.33

For incubation mixture, see Materials and Methods. The amount of poly-4-thiouridylic acid used was 1 μ g.

In all the experiments described above the total amount of poly-ribosomes used in the incorporation assay was 1.0 A_{260} unit. From the results shown in Table I, the degree of the inhibition is dependent on the ratio of inhibitor to polyribosomes. By increasing the amount of polyribosomes from 0.5 A_{260} unit to 3.0 A_{260} units (with a constant amount of poly-4-thiouridylic acid) the percent of inhibition is reduced from 90 to 11. In Table I the quantitative binding of poly-4-thiouridylic acid to ribosomes is calculated: 1 μ g of poly-4-thiouridylic acid is bound to about 0.4 A_{260} units of ribosomes.

Table II: The incorporation of phenylalanine into polyphenylalanine as a function of poly U concentration.

Polyuridylic acid added (μ g)	Incorporation of [14 C]phenylalanine (cpm)	
	A	B
20	470	40
40	550	40
80	590	60
160	560	60
200	460	70
400	310	160
800	200	0
1600	60	0

Incubation mixtures - see Materials and Methods. The amount of poly-4-thiouridylic was 1 μ g; polyribosomes - 0.5 A_{260} . Incubation time was 15 min. The endogenous incorporation values were subtracted from the total value. A, in the absence of poly-4-thiouridylic acid; B, in the presence of poly-4-thiouridylic acid.

The effect of different ratios of poly U/poly 4-thio-U on the incorporation of phenylalanine into polyphenylalanine was tested. When the ratio of poly U to poly-4-thio U was between 20 to 200, the incorporation of phenylalanine was inhibited almost completely. However, when the ratio of poly U to poly-4-thio U was 400, the inhibition was 50% only. Higher incorporation values could not be achieved because of the inhibitory effect caused by high concentrations of poly U (see Table II, Column A).

These results show that the affinity of ribosomes to poly-4-thio U is much greater than the affinity of ribosomes to poly U.

Table III: The stability of the poly-4-thiouridylic acid-ribosomes complex			
Preincubation with	Incorporation of [^{14}C]Phe (cpm)		
	None	addition	Poly U
no addition	281		795
Poly U 400 μg	388		938
Poly-4-thio U 40 μg	376		877
Poly-4-thio U 400 μg	338		942
Polyribosomes (36 A_{260} units) were incubated for 30 min in 2 ml of Tris HCl pH 7.4, 60 mM, KCl 50 mM, MgCl_2 10 mM and the additions noted in the table. After incubation the mixtures were spun for 90 min at 100,000 x g. The pellet was suspended and assayed for incorporation.			

The stability of the ribosomes-poly-4-thiouridylic acid complex was tested. From the data shown in Table III it can be seen that this complex does not stand centrifugation, and that the inhibitory effect is reversible under the experimental conditions used.

It can be concluded therefore, that the inhibition of the incorporation of [^{14}C]phenylalanine into hot 5% TCA insoluble material

by poly-4-thiouridylic acid is due to the binding of the poly-4-thiouridylic acid to the ribosome.

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