INTRAMOLECULAR CROSS-LINKING OF SINGLE-STRANDED COPOLYMERS OF 4-THIOURIDINE AND CYTIDINE

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Highly polymerized copolymers of 4-thiouridine and cytidine were prepared by direct thiolation of polycytidylic acid. Irradiation with 300-400 nm light of these copolyribonucleotides results in the covalent linkage of about 10% of the thiouridines with cytidines. No such photoreaction occurs with the corresponding mixture of nucleosides, nor with either the thiolated CpC dinucleotide or the double-stranded complexes formed by the copolymers when associated with poly(I) or poly(G) : the thiouridine-cytidine covalent links are formed between non adjacent residues in the folded single-stranded chains.

Irradiation at 335 nm of E. coli $tRNA_1^{Val}$ results in the specific and quantitative cross-linking of the s^4U* residue in position 8 in the sequence with a C in position 13 (1,3) yielding the Pdo(4-5)Cyd photoproduct (4,5). This photoreaction was found to be a valuable tool for studying the tRNA structure in solution (see ref 6,7 for discussion). An important feature is that formation of the 8-13 link is quantitative in the native and related conformational states of $tRNA_1^{Val}$; little or no photoproduct is obtained in the denaturated state of the molecule or in E. coli tRNA fragments containing the s^4U and C residues in position 8 and 13 respectively (8, 9). The same conclusion is valid for mixed E. coli tRNAs : however some adduct can be obtained in conditions where the tRNA is unfolded (10).

*Abbreviations : s^4U=4-thiouridine; C=cytidine; CpC=the dinucleotide 3'5' monophosphate; poly(C)=polycytidylic acid; poly(I)=polyinosinic acid; poly(G)=polyguanylic acid; Pdo(4-5)Cyd=the diriboside of 5-(4'-pyrimidin-2'-one) cytosine.

This raises the question of whether such covalent links can be induced in a polynucleotide chain containing the s^4U and C residues. Using copoly(s^4U,C) synthetized with polynucleotide phosphorylase POCHON et al. (11) failed to detect any Pdo(4-5)Cyd formation. This is unexpected since the photoproduct is obtained by irradiation of a mixture of s^4U and C in appropriate conditions and since some transient interactions between distinct parts of a single-stranded chain should occur.

We have developed a new procedure for the synthesis of poly(s^4U,C) and reexamined the formation of the adduct in this polymer. We present here our preliminary results concerning:

i) the direct conversion of C into s^4U in poly(C) and CpC using the amino-thiol exchange reaction described by UEDA et al. (12, 13) and the subsequent characterization of the samples.

ii) the photochemical behaviour of s^4U in these copoly(s^4U,C) samples that leads to conclusions opposite to those of POCHON et al. (11).

MATERIAL AND METHODS

Products : Poly(C), poly(I) and poly(G) were Miles Elkehart products. The dinucleotide CpC was a kind gift of Dr. A.M. Michelson.

Physical techniques : The apparatus used for absorption and fluorescence measurements have been described elsewhere (5). All irradiation experiments were performed with the Cunow lantern equipped with an MTO 324a filter that cuts off light of wavelengths shorter than 315 nm. The irradiated cuvette was placed in a thermostated (25°C) cell holder. Sedimentation coefficient determinations were performed in a Beckman type E ultracentrifuge equipped with a photoelectric scanner in a solvent containing 0.1 M Tris buffer pH 7 and 1 M NaCl.

The molar extinction coefficients used were (Mole^{-1}. cm^{-1}) 17,000 at 330 nm for s^4U at pH 7 (14) 9,000 at 270 nm for cytidine; 14,000 at 330 nm and 20,000 at 380 nm respectively for Pdo(4-5)Cyd and its sodium borohydride reduction product at pH 9.7.

Other procedures : Pancreatic RNase digestion was performed by adding 10 µl of a 1mg/ml enzyme solution per ml of sample followed by incubation at pH 7 for one hour at 25°C.

The amino-thiol exchange reaction was performed as follows: to 3 ml of a water-pyridine (1/1) solution containing 2 mg of poly(C) and cooled to -70°C is added 1 ml of liquid H_2S. The solution is abandoned in a steel container at room temperature and H_2S is removed when the

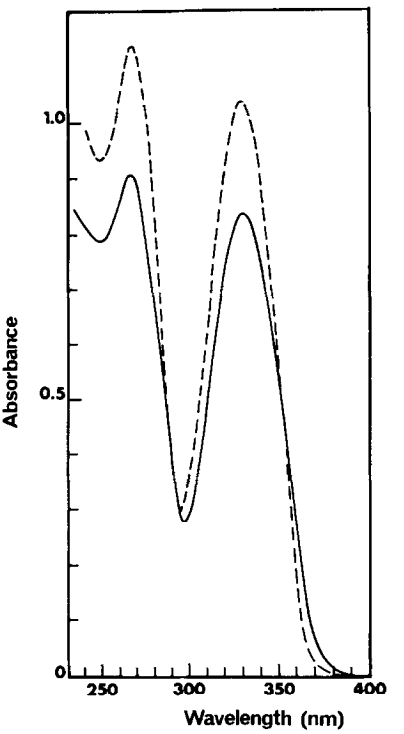

Fig. 1 : Absorption spectra of intact —— and RNase digested --- poly(s^4U,C) containing 36% s^4U; solvant was 0.1 M sodium cacodylate buffer pH 7.

reaction time is elapsed. The polynucleotide is precipitated by addition of 2N NaCl solution and 30 ml of ethanol and kept overnight at -20°C. The precipitate is centrifuged, washed with ethanol, taken up in water and lyophilized. Sodium borohydride reduction (see ref 15).

Definitions : A^{330} unit : the quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 330 nm when measured in a 1 cm path-length cell.

RESULTS

1) Preparation and characterization of the s^4U containing dinucleotides and polynucleotides: By applying the procedure of UEDA et al. (12, 13) to poly(C) and CpC and varying the reaction time we have obtained samples with different s^4U contents.These samples were characterized by their UV absorption spectra since s^4U and C absorb in widely distinct regions (the λ^{max} are at 330 nm and 270 nm respectively at neutral pH). As shown in Fig. 1 degradation of poly(s^4U,C) at the nucleotide level results in a hyperchromic effect at both 270 and

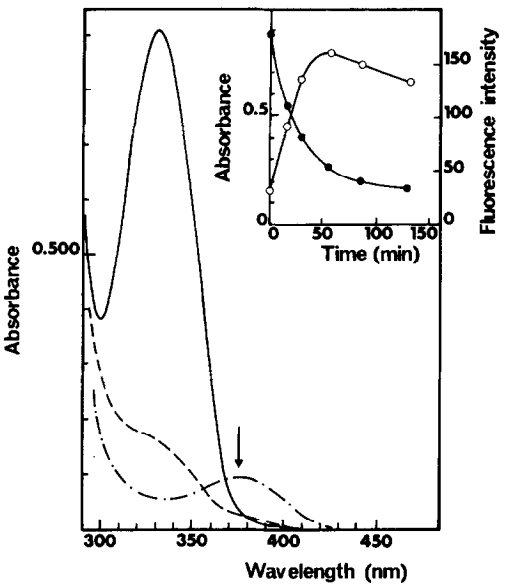

Fig. 2 : Introduction of Pdo(4-5)Cyd in poly(s^4U,C) (36% s^4U). The polymer was irradiated at 25°C in 0.1 M sodium cacodylate pH 7. The figure shows the initial absorbance spectrum of the sample —— its spectrum after 130 min of irradiation and addition of NH_3 to pH 9.7 ---, and finally after reduction with sodium borohydride for 120 min at pH 9.7 —•—. In the insert is represented the course of s^4U photolysis during the irradiation as followed by absorbance at 330 nm -•-•- and fluorescence of the $NaBH_4$ reduced polynucleotide measured at pH 9.7 on 50 µl aliquots taken at appropriate times during the photoreaction o-o-o.

330 nm, typical of a single-stranded chain : similar effects are obtained with poly(C) at 270 nm or with enzymatically synthetized poly(s^4U,C) and with poly(s^4U) at 330 nm (16, 17).

As already established at the mono and dinucleotide level (12, 13) the amino-thiol exchange reaction occurs without other modifications of the bases. This is true at the polynucleotide level since the absorption spectra of our RNase digested samples can well be accounted for by the appropriate contributions of the spectra of s^4U and C. Moreover no extensive chain cleavage occurs : the S_{w20} values found for intact poly(C) and two copoly(s^4U,C) samples containing 2% and 36% s^4U were 13, 6.6 and 6S respectively.

The characteristic luminescence of s^4U in water (1,11,18,19) is present in thiolated CpC with a two fold reduced quantum yield but cannot be detected in poly(s^4U,C).

The s^4U content of the samples can be easily deduced from the absorption data (Table 1). Obviously thiolation of CpC yields a mixture of dinucleotides. On the basis of random conversion one

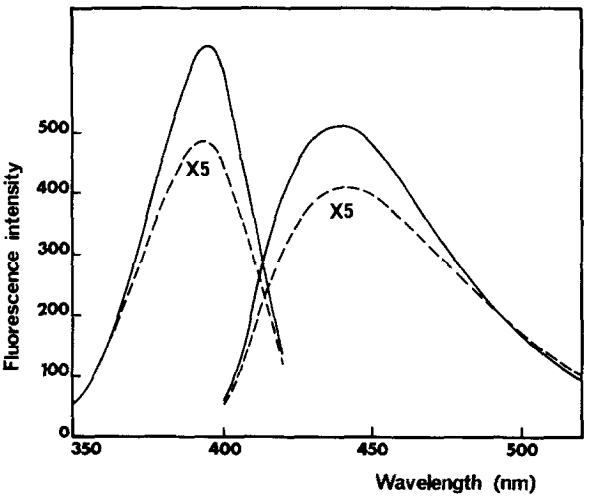

Fig. 3 : Excitation (left) and emission (right) uncorrected spectra of reduced Pdo(4-5)Cyd in E. coli tRNA (0.025 A^{380} units) and poly(s^4U, C) containing initially 8% s^4U (0.055 A^{380} units). The irradiated and reduced samples were dialyzed against 0.1 M sodium cacodylate buffer and fluorescence measurements performed in the presence of 3.10^{-2} M $MgCl_2$ at 25°C (λexc 380 nm λem 450 nm).

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should find : CpC 36%, s^4Ups^4U 16%, and s^4UpC plus Cps^4U 48%.

2) Light induced cross-link formation in copoly(s^4U,C). Formation of Pdo(4-5)Cyd can be easily detected using the known spectral data (15) of its sodium borohydride reduction product (5-20). Indeed a new absorption band (λ^{max} 380 nm) appears (Fig. 2) after reduction of the irradiated poly(s^4U,C) samples. To this absorption band is associated the characteristic fluorescence (λ^{max}_{ex} 385 nm - λ^{max}_{em} 445 nm) (Fig. 3). At pH 7, 25°C, in the presence of 10^{-2} M Mg^{++} the emission proceeds with a quantum yield of 2% close to that observed in the oligonucleotides resulting from the T_1 RNase digestion of mixed tRNA (21). Since the molar extinction coefficients at 330 nm of s^4U and Pdo(4-5)Cyd are close to each other (3) our data - insert of Fig. 2 - show that the formation of the adduct is in competition with other photoprocesses. Furthermore in poly(s^4U,C) the adduct is photolyzed as already observed in the isolated state (5). Under the conditions of Fig. 2 the maximum yield of conversion of s^4U into Pdo(4-5)Cyd is close to 10% and looks very similar in the different poly(s^4U,C) samples examined here. The quantum yield for its formation is about ten times lower than observed in E. coli $tRNA_1^{Val}$. No evidence for chain cleavage or chain aggregation can be found : the sedimentation coefficient , of a poly(s^4U,C) sample (containing 36% s^4U) is close

TABLE I

Composition and absorption characteristics of the copoly(s^4U,C) and dinucleotide samples

Thiolated sample	Poly(C)			CpC
Time (hours) of H_2S treatment	24	72	144	144
$\frac{A^{270}}{A^{330}}$ in thiolated samples	21	6.2	0.93	0.90
$\frac{A^{330}\ \text{degraded}}{A^{330}\ \text{intact}}$ in thiolated samples	1.28	1.34	1.23	1.07
s^4U content %	2.2	7.7	36	41

Measurements were performed at 25°C in 0.1 M sodium cacodylate pH 7 buffer containing 0.1 M NaCl. Digestion of samples was performed as described in Methods. The concentrations in s^4U and C were deduced from their respective contribution to the absorbance of the digested samples using the extinction coefficients listed in Material and Methods.

to 6 and remains unchanged when 38% and 75% of the s^4U residues are photolyzed.

Although the adduct is readily obtained in irradiated single-stranded poly(s^4U,C), very low amounts, if any, are detected in:

i) the RNase digest of poly(s^4U,C) (Table 2).

ii) the mixture of dinucleotides obtained by H_2S treatment of CpC (Table 2).

iii) the double-stranded structures formed between a poly(s^4U,C) sample containing 8% of s^4U and poly(G) or poly(I). (The evidence for formation of the complexes and their characterization will be presented elsewhere).

DISCUSSION

In order to reexamine the photochemical behaviour of s^4U in copoly(s^4U,C) we have applied the procedure of UEDA *et al.* (12) to poly(C) and prepared three highly polymerized copoly(s^4U,C) samples (Table 1). The absorption and luminescence spectral data of these polymers are very similar

TABLE II

Formation of the thiouridine-cytidine photodimer in single-stranded copoly(s^4U,C) containing 36% s^4U and in the thiolated "dinucleotide".

	Poly s^4U,C	digested Poly(s^4U,C)	"dinucleotide"
ΔA^{380}	0.280	0.00	0.00
IF	3,700	97	60
IFo	70	40	45

Initial absorbances at 330 nm were respectively 1,500 and 1,850 for the intact and digested polymer and 1,430 for the dinucleotide. The samples were irradiated in a solvent containing 0.1 M sodium cacodylate pH 7 and 1 M NaCl, at 25°C for 70 min and subsequently reduced by sodium borohydride. ΔA^{380} is the absorption difference measured at 380 nm for the irradiated samples before and after reduction. The fluorescence (λexc 390 nm λem 450 nm) was measured with both the irradiated and reduced (IF), and the control non irradiated and reduced (IFo) samples (in the conditions used here the pancreatic RNase digestion of the irradiated and reduced poly(s^4U,C) sample decreases the fluorescence signal by a factor of 3).

to those previously reported for an enzymatically synthetized sample (11). The chemical procedure for the conversion of C into s^4U at the polynucleotide level should be very useful in view of the current interest in the physical properties of s^4U containing polynucleotides (22).

As judged by absorption measurements, the photochemical behaviour of s^4U is also quite similar in the chemically (Fig. 2) and enzymatically synthetized samples. By themselves these data do not rule out the formation of Pdo(4-5)Cyd as wrongly concluded by POCHON et al. (11). Indeed direct evidence has been obtained here for the photochemical conversion of s^4U into this adduct with a 10% yield. A salient question is whether those links are formed between s^4U and C residues present in the same chain or between distinct chains ? The latter possibility can be rejected on the following basis :

i) the sedimentation velocity of poly(s^4U,C) remains practically unaltered during the course of s^4U photolysis thus eliminating the eventuality of an aggregation.

ii) at the nucleoside concentrations used here (less than 5.10^{-4}M) the photoadduct is only obtained in the intact single-stranded chain but not with the corresponding mixture of nucleosides (Table 2). Irradiation of s^4U in single-stranded poly(s^4U,C) therefore results in the formation of

intramolecular cross-links. Since no adduct is formed in the dinucleotide - the simplest unit of a polynucleotide chain - the links are certainly formed between non-adjacent residues. This leads to the following dynamic picture: in solution the single-stranded chain folds on itself and some distant s^4U and C residues come transitorily in the vicinity of each other, thus providing the appropriate geometry for adduct formation. This interpretation is further supported by the lack of Pdo(4-5)Cyd formation in poly(I).poly(s^4U,C) and poly(G).poly(s^4U,C) complexes : the poly(s^4U,C) chains are firmly and rigidly maintained by the hydrogen bonding of the cytidines (and possibly of some s^4U) to the purines of the complementary chain. We can therefore safely conclude that in poly(s^4U,C) the covalent links are formed between non adjacent residues of the chain. The minimum distance necessary for this formation - a measure of chain flexibility - is not yet known. Notice that in the native conformation of tRNA where strong structural constraints certainly occur, the cross-linked bases are five residues apart (1-2).

In spite of extensive studies on polynucleotide photochemistry (23) no photoreaction resulting in a linkage between non adjacent residues inside a single-stranded chain has, to our knowledge, been described or even postulated. Such photoreactions may not be exceptional : it is known that cyclobutane-type dimers arise from adjacent pyrimidines but not excluded that they can derive from more distant residues. The method developed here (conversion of C into s^4U and the subsequent cross-linking of s^4U) can be applied to other nucleic acids to help explore their structure in solution.

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