INTRAMOLECULAR ALKYLATION OF tRNA BY A NITROGEN MUSTARD BOUND COVALENTLY TO AN AMINO ACYL RESIDUE

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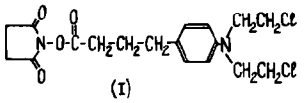
1. Introduction

A promising approach to studies of the tertiary structure of tRNA seems to us to be the application of reagents containing two reactive moieties – one highly reactive group enabling covalent binding of the reagent to "peculiar point" of the macromolecule (terminus of native molecule or large fragment, unique base, amino acyl residue), and another, less reactive group that could non-selectively modify the neighbouring monomer units in the three-dimensional structure.

N-hydroxysuccinimide ester of chlorambucil has been investigated as such a reagent in the course of the present studies:



(I)

This reagent was used to attach an alkylating nitrogen mustard moiety to the amino acyl residue of an aminoacyl-tRNA *.

2. Materials and methods

The isolation of unfractionated tRNA from baker's yeast [3] and the method of its enzymatic amino-acylation with [^{14}C] L-valine (180 Ci/mole, Chemapol, USSR) [4] have been described. BDEAE-cellulose † [5] was kindly given by Dr. V.F. Podgorny of this laboratory. *N*-hydroxysuccinimide (m.p. 99–101°) was synthesized according to [6]. Chlorambucil, kindly supplied by Dr. O.V. Kildisheva (Institute of Elementoorganic Compounds, Moscow), was recrystallized from benzene (m.p. 64–66°). Radioactivities were counted with a Nuclear Chicago Mark 1 scintillation counter.

The synthesis of *N*-hydroxysuccinimide ester of chlorambucil was performed by a slight modification of the general method of Anderson et al. [6]. 288 mg dicyclohexylcarbodiimide in 2 ml dry tetrahydrofuran was added to 431 mg chlorambucil and 166 mg *N*-hydroxysuccinimide in 12 ml of the same solvent and the mixture left overnight at 25°. The precipitate of dicyclohexylurea was filtered off, the filtrate concentrated by evaporation was again filtered, and the precipitate washed with tetrahydrofuran and 4–5 vol of pentane were added to the combined filtrates. Under refrigeration, crystalline chlorambucil *N*-hydroxysuccinimide ester was obtained, m.p. 83–85°, yield 64%. The substance contained no ionic chlorine. Analysis, calculated: C 53.87, H 6.49, N 6.98, O 15.96, Cl 17.70; found: C 54.41, H 5.49, N 7.22, O 15.66, Cl 17.14 %. IR-spectrum contains bands at 1220 and 1750 cm^{-1} (ester and carbonyl, respectively).

Acylation of the amino acyl residue of [^{14}C]valyl-tRNA was performed essentially according to

* Our reagent has been applied earlier in this laboratory to acylate the phenylalanine residue of phenylalanyl-tRNA^{Phe} for subsequent affinity labelling of ribosomes [1, 2].

† BDEAE-cellulose, benzoyldiethylaminoethylcellulose.

Hamburger et al. [7]. To a solution of 1–3 A_{260} units of [^{14}C] valyl-tRNA (unfractionated) in 2 μl of water were added 2 mg chlorambucil *N*-hydroxysuccinimide ester in 45 μl dimethylsulfoxide, 5 μl 0.25 M triethylammonium sulfate pH 8.1 and 1 μl 1 M $Na_2S_2O_3$. The mixture was kept for 2 hr at 30° and diluted with 1.5 ml 0.1 M NaCl–0.02 M $MgSO_4$–0.01 M Tris-HCl, pH 7.2. The precipitate of the reagent was removed by extraction with ether (3 × 5 ml) and the aqueous layer applied to Sephadex G-50 "fine" column equilibrated with 0.1 M NaCl–0.02 M $MgSO_4$–0.01 M Tris-HCl pH 7.2. Elution was performed by the same buffer at 4°. The extent of the acylation of the valine residue of valyl-tRNA^{Val} was determined by subjecting the polymer fraction (0.3–1 A_{260} units per ml) to hydrolysis catalyzed by Cu^{2+} ions [8]; it was 80%.

Intramolecular alkylation of tRNA^{Val} by the nitrogen mustard moeity attached to the valine residue was performed by incubation of the polymer fraction obtained in the above experiment at 25°. To determine the extent of alkylation, 5 μl of crystalline pancreatic ribonuclease solution (0.1 mg/ml) was added to a 1 ml aliquot of the said polymer fraction, the mixture incubated for 15 min at 37°, diluted 10 times with water and applied to a DEAE-cellulose column (0.5 ml). The column was washed with 0.01 M NaCl in 7 M urea – 0.02 M Tris-HCL, pH 7.5 till the absence of radioactivity in the effluent and then with 1 M NaCl in the same solvent. The relative radioactivity of the fraction eluted with 1 M NaCl was assumed to represent the extent of intramolecular alkylation. Control experiments revealed that under these conditions no radioactivity of [^{14}C] valyl-tRNA is retained by the column after ribonuclease treatment, and that the first buffer completely elutes authentic chlorambucilyl-[^{14}C]-valine. Kinetics of the intramolecular alkylation are shown in fig. 2.

Other procedures are described in the legends to the figures.

3. Results and discussion

It is evident that the success of the application of the method outlined above with *N*-hydroxysuccinimide chlorambucil ester depended first of all on the

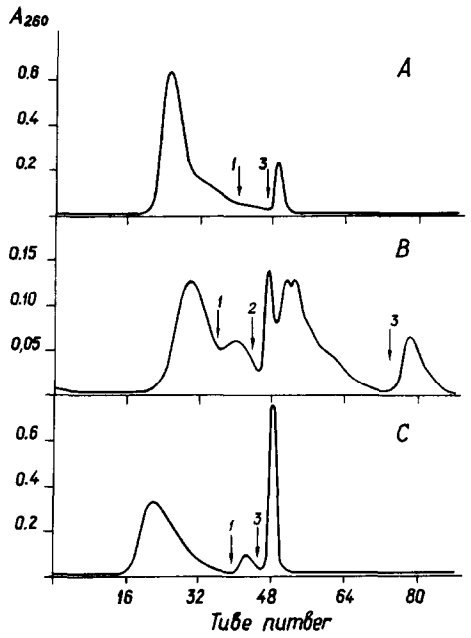


Fig. 1. Chromatography of tRNA preparations on BDEAE-cellulose. Column 0.3 × 15 cm, linear gradients of total volume 40 ml, elution at 2 ml/hr, fraction volume 0.9 ml. Before arrow 1 – elution with sodium chloride gradient 0 to 1 M; at arrow 1 – elution with 1 M NaCl; arrow 2 – elution with dioxan gradient 0 to 10% on 1 M NaCl background; arrow 3 – elution with 10% dioxan – 1 M NaCl.
A) Unfractionated yeast tRNA, 14 A_{260} units;
B) non-aminoacylated unfractionated yeast tRNA treated with chlorambucil *N*-hydroxysuccinimide under the acylation conditions (see Materials and methods) but in the absence of thiosulfate;
C) the same as in B) but treatment under the usual conditions of acylation in the presence of thiosulfate.

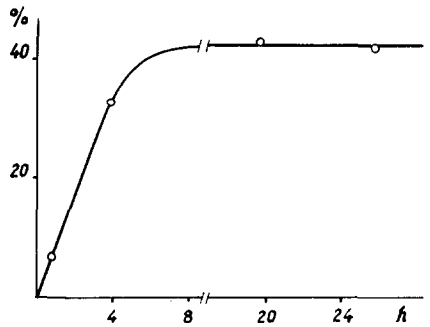
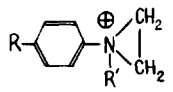


Fig. 2. Kinetics of the intramolecular alkylation (see text). Ordinate: extent of intramolecular alkylation, %.

possibility of binding the nitrogen mustard moiety to a "peculiar point" on the macromolecule – its amino acyl residue – under conditions which did not allow any significant intermolecular alkylation in the course of the acylation. The second task was to transfer the acylated aminoacyl-tRNA into an appropriate buffer solution as soon as possible after the acylation in order that intramolecular alkylation took place under conditions of stability of the naive tertiary structure.

To evaluate the content of intermolecularly alkylated tRNA we applied chromatography on BDEAE-cellulose since it is known that addition of even a single hydrophobic substituent to tRNA greatly increases its affinity for this sorbent [9, 10]. The model experiment was performed with non-aminoacylated unfractionated tRNA; it was incubated under the acylation conditions as described in "Materials and methods" but in the absence of sodium thiosulfate. It appeared that under these conditions some intermolecular modification of tRNA does take place as revealed by a shift of about 50% of the absorbancy to fractions with increased affinity for BDEAE-cellulose (cf. fig. 1A and fig. 1B). However, this side reaction did not take place to any significant extent when sodium thiosulfate was present in the reaction mixture. The amount of A_{260} units in the peak eluted with 10% dioxan – 1 M NaCl in this control experiment was about 15% (cf. 6% with native tRNA, fig. 1A and 1C).

It was found in this laboratory that the efficiency of the alkylation of nucleic acids with nitrogen mustards depends to a great extent on the local increase in concentration of the reactive ethyleneimmonium cations



nearby nucleic acid polyanion [11]. For example, efficiency of the alkylation of tRNA by a nitrogen mustard with a positively charged R-radical appeared very high even at high ionic strengths and in the presence of Mg^{2+}, unlike that with mustards whose R-radical is uncharged [12]. Hence, it was anticipated that the efficiency of the intramolecular alkylation of tRNA will be high due to attraction of covalently

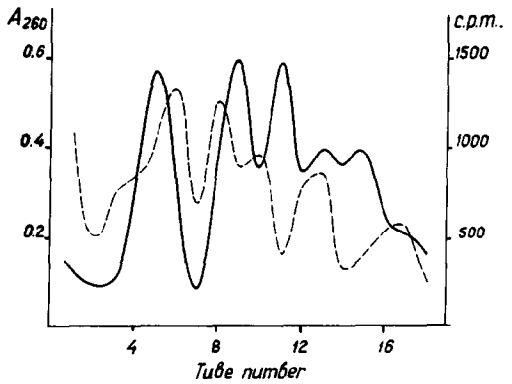


Fig. 3. Chromatography of the pancreatic RNAase digest of intramolecularly alkylated tRNA on DEAE-cellulose. 0.3 mg intramolecularly alkylated tRNA, 2.2 mg of unfractionated tRNA (carrier) and 25 μg pancreatic ribonuclease were incubated for 18 hr at 23° in 1 ml 0.06 M Tris-HCl, pH 7.5. The digest was applied to a DEAE-cellulose column (0.5 × 16 cm) after 5-fold dilution. Elution with linear gradient of sodium chloride (0 to 0.5 M) in 7 M urea – 0.02 M Tris HCl pH 7.5 (40 ml), rate of elution 1 ml/hr, fraction volume 0.9 ml. (————) A_{260}; (– – – – –) radioactivity, cpm/ml.

bound reactive ethyleneimmonium moiety by adjacent region of tRNA molecule with high density of negative charges.

It is seen in fig. 2 that the efficiency of the intramolecular alkylation of tRNA was in fact high (40%) in spite of the high concentration of shielding sodium and magnesium cations necessary for maintaining the native conformation.

The last problem investigated preliminarily in the present studies was the specificity of the intramolecular alkylation. Fig. 3 shows the chromatographic pattern of intramolecularly alkylated tRNA pancreatic RNAase digest. It is seen that the 40% of radioactivity that is due to intramolecular alkylation is not present in an individual oligonucleotide. This is not surprising for three reasons. First of all, the substance studied was unfractionated tRNA containing several isoacceptor valine-tRNA's. Secondly, there are two alkylating groupings in the chlorambucilyl residue. And, last, molecular models demonstrate that the distance from the ethyleneimmonium cation and the ester bond between the valine and the adenosine residues varies in the limits 0 to 15 Å; hence, according to models proposed for the tertiary structure of tRNA (for review, see [13]), a large region of the macromolecule appears potentially reactive.

References

[1] E.S. Bochkareva, V.H. Budker, A.S. Girshovich, D.G. Knorre and N.M. Teplova, FEBS Letters 19 (1971) 121.
[2] E.S. Bochkareva, V.G. Budker, A.S. Girshovich, D.G. Knorre and N.M. Teplova, Dokl. Akad. Nauk SSSR 201 (1971) 352.
[3] L.S. Sandakhchiev, V.K. Starostina, L.E. Stefanovich and V.M. Chuchaev, Molekul. Biol. 1 (1967) 463.
[4] M.A. Grachev, N.I. Menzorova, L.S. Sandakhchiev, E.I. Budowsky and D.G. Knorre, Biokhimiya 31 (1966) 840.
[5] I. Gillam, S. Millward, D. Blew, M. von Tigerstrom, E. Wimmer and G.M. Tener, Biochemistry 6 (1967) 3043.
[6] G.M. Anderson, J.E. Zimmerman and F.M. Callahan, J. Am. Chem. Soc. 86 (1964) 1839.
[7] A.D. Hamburger, N. DeGroot and Y. Lapidot, Biochim. Biophys. Acta 213 (1970) 113.
[8] P. Shofield and P.C. Zamecnik, Biochim. Biophys. Acta 155 (1968) 410.
[9] I.H. Maxwell, E. Wimmer and G.M. Tener, Biochemistry 7 (1968) 2629.
[10] I. Gillam, D. Blew, R.C. Warrington, M. von Tigerstrom and G.M. Tener, Biochemistry 7 (1968) 3459.
[11] N.I. Grineva, V.F. Zarytova, D.G. Knorre and V.A. Kurbatov, Dokl. Akad. Nauk SSSR 194 (1970) 331.
[12] N.I. Grineva, D.G. Knorre and V.A. Kurbatov, Dokl. Akad. Nauk SSSR 201 (1971) 609.
[13] F. Cramer, Progr. Nucleic Acids Res. Mol. Biol. 10 (1971) 391.