EFFECT OF AURINTRICARBOXYLIC ACID AND OF NaF ON THE BINDING OF GLOBIN MESSENGER RNA TO RETICULOCYTE 40S RIBOSOMAL SUBUNITS.

B. Lebleu, G. Marbaix, J. Wérenne, A. Burny and G. Huez

Laboratory of Biological Chemistry,
Faculty of Sciences,
University of Brussels, Belgium.

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Summary : Purified globin messenger RNAs bind selectively to 40S ribosomal subunits from reticulocytes in an energy-free system. Attachment is totally inhibited by aurintricarboxylic acid whereas NaF does not prevent binding.

In bacteria, ribosomes dissociate after each cycle of protein synthesis and subunits are necessary for the initiations of new chains (1-3). A similar situation probably prevails in eukaryote cells (4-8). The interaction of mRNA and 40S ribosomal subunits as the first event of the initiation process has been demonstrated by Heywood using mRNAs purified from embryonic muscle (9). We made similar experiments using mRNAs from rabbit reticulocytes which carry the information for globin chains (10-12). Our results clearly show that this mRNA binds selectively to the light ribosomal subunit.

We have used this system to obtain further information on the mode of action of two inhibitors of initiation of protein synthesis : NaF and aurintricarboxylic acid (ATA).

It is established that NaF inhibits the dissociation of ribosomes in animal cells (7, 13). Since on the other hand mRNA remains attached to single 80S ribosomes in reticulocytes (14)

or HeLa cells (7) incubated with NaF, it can be assumed that mRNA is able to bind to ribosomes in the presence of this inhibitor. The experiments reported in the present paper afford direct evidence that NaF does not inhibit the binding of mRNA to 40S ribosomal subunits.

Aurintricarboxylic acid is known to specifically hinder the binding of a phage mRNA to E. coli ribosomes without affecting the elongation of polypeptide chains (15). The data presented here show that binding of globin mRNA to 40S ribosomal subunits from reticulocytes is totally inhibited by ATA in conditions under which the incorporation of amino acids in polypeptide chains is not drastically reduced.

Materials and methods.

Ribosomal material.

Reticulocytes were obtained as previously described (10). The cells were lysed at 4°C by shaking in four volumes of hypotonic saline solution (0.01 M Tris-HCl pH : 7,4; 0.01 M KCl, 0.001 M Mg acetate) for two minutes and the isotonicity was then restored by one-tenth volume of 1.5 M NaCl. Ribosomes and ribosomal subunits were pelleted from the stroma-free lysate by a 15 hour centrifugation at 78.000 g and 4°C.

Preparation of the ^{32}P-labelled 9S-messenger RNA.

The ^{32}P-mRNA for globin chains was released from polyribosomes by EDTA-treatment as a ribonucleoprotein complex (mRNP) (16). Labelling was obtained by injecting anaemic rabbits subcutaneously with 10 mc of ^{32}P-orthophosphate 15 hours before sacrifice.

Binding test.

Ribosomes (10 mg) were incubated for 10 minutes at 25°C with

20 µg of ^{32}P-labelled mRNP (150 cpm/µg of RNA) in 1,5 ml of the medium A described by Heywood (9) (0.02 M Tris-HCl pH : 7,6, 0.005 M Mg acetate, 0.15 M KCl, 0.006 M mercaptoethanol).

Aurintricarboxylic acid (ATA) and NaF were added to a final concentration of 0.0002 M and 0.01 M respectively before addition of the labelled mRNP. After incubation, the reaction mixtures were rapidly cooled, layered on top of 15-30 % (w/v) linear sucrose gradients made in medium A and spun for 12 hours at 17.500 rpm and 5°C in the SW 27 rotor of a Spinco ultracentrifuge. Fractions of the gradients were collected by injecting 40 % saccharose through the bottom of the tubes and their absorbance was determined at 260 nm. Samples were filtered through nitrocellulose membrane filters (Millipore Ha, 0,45 µ); the filters were then washed with 3 ml of buffer A, dried and their radioactivity was determined by liquid scintillation counting.

Effect of ATA on a subcellular protein synthesizing system from reticulocytes.

The effect of ATA on protein synthesis was tested in an "in vitro" system from reticulocytes slightly modified from Lamfrom (17) : 3.36 µmoles Tris-HCl pH : 7,6, 4.2 µmoles Mg acetate, 4.8 µmoles KCl, 1.2 µmoles NH_4Cl, 2 µmoles mercaptoethanol, 2 µmoles ATP, 5 µmoles PEP, 0.4 µmoles GTP, 0.125 µmoles of each amino acid except the labelled one, 200 µg of pyruvate kinase and 0.6 ml of a 1/4 cell lysate in a final volume of one ml. In such a system, most incorporation reflects elongation but new chains can be initiated. Samples were precipitated with trichloroacetic acid, boiled for 20 minutes and filtered through Whatman glass filter papers. The filters were dried and counted in a gas-flow counter. Aliquots of the system were quickly cooled and analyzed by centrifugation on 15/30 % (w/v) linear sucrose

gradients made in lysis solution. The gradients were spun at 4°C for 50 min at 49.000 rpm in the SW 50 rotor of a Spinco ultracentrifuge and their absorbance at 260 nm was continuously recorded with a Gilford spectrophotometer.

Results and discussion.

It will be reported elsewhere that the binding of the 9S mRNA to the 40S ribosomal subunits is more efficient when this messenger RNA is included in the ribonucleoprotein complex (mRNP)

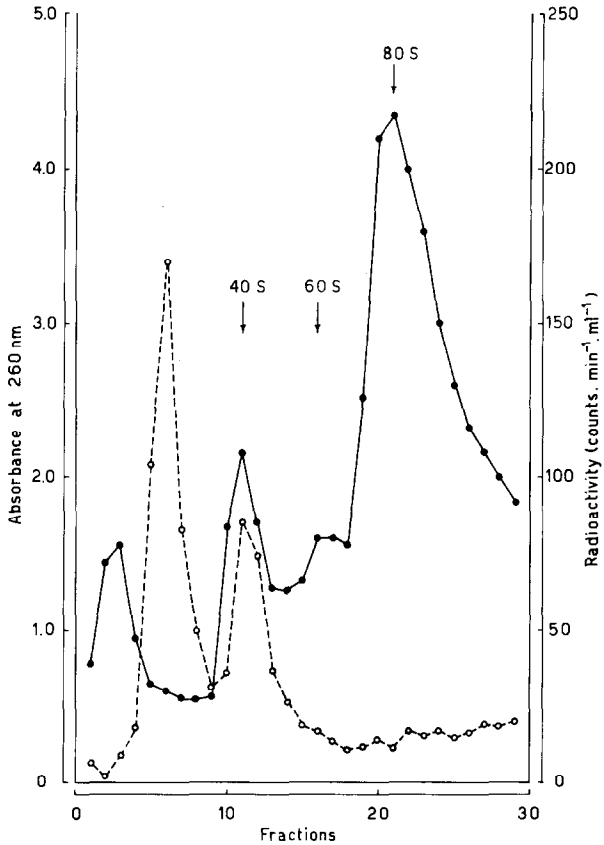

Fig. 1. Sedimentation profile in a sucrose gradient of ribosomes incubated with ^{32}P labelled mRNP. For details, see Materials and Methods.

o —— o Absorbance at 260 nm.
o --- o ^{32}P activity retained on nitrocellulose membrane filters.

that we have isolated and characterized (18, 19) than when it is completely deproteinized. This is the reason why we use mRNP complexed mRNA in this study.

Fig. 1 shows the sedimentation profile in a sucrose gradient of a ribosomal suspension which was incubated with ^{32}P-labelled mRNP. It will be noticed that a large fraction of mRNA is found selectively associated to the 40S ribosomal subunit and not at all with other particles. This result, obtained with natural, well-defined messengers (the mRNAs for globin chains) fits perfectly with those reported by Heywood using the mRNAs for embryonic chick muscle proteins. As Lockhard and Lingrel (20) showed that the mRNA 9S used in this study can be translated to globin chains in a subcellular system from reticulocytes, the observed binding is probably the first step of initiation.

Fig. 2 show the result of an experiment similar to those reported in Fig. 1 except that NaF 10^{-2}M was already present in the incubation medium before addition of the labelled mRNP. It is perfectly clear that NaF 10^{-2}M does not hinder the attachment of messenger RNA to the light ribosomal subparticle.

Fig. 3 shows that aurintricarboxylic acid (ATA) 2.10^{-4}M entirely prevents the attachment of the mRNA to the 40S subparticles. The two inhibitors therefore act at two different steps of initiation. Grollman et al. have shown that ATA 5.10^{-5}M inhibits the formation of the complex "phagic RNA-E.coli 70S ribosomes" (15) and the binding of polyuridylic acid to reticulocytes ribosomes (21). Marcus et al. have similarly demonstrated that the initiation complex between wheat embryo ribosomes and tobacco mosaïc virus (TMV) RNA is not formed in the presence of ATA 5.10^{-5}M (22). Using hemoglobin messenger RNA, we have thus established, in the case of mammalian ribosomes, the step

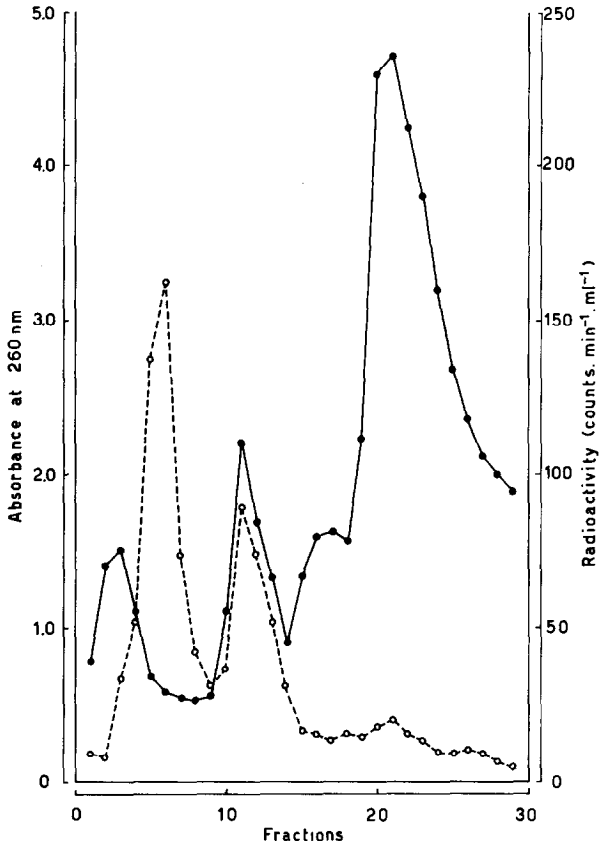

Fig. 2. Sedimentation profile in a sucrose gradient of ribosomes incubated with ^{32}P-labelled mRNP and 0.01 M NaF. For details, see Material and Methods.

o —— o Absorbance at 260 nm.
o --- o ^{32}P activity retained on nitrocellulose membrane filters.

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at which this inhibition occurs, i.e. by hindering the binding of the mRNA to the light ribosomal subunit. ATA thus inhibits the very first step of initiation. On the other hand, the elongation of polypeptide chains seems not to be greatly affected at the concentration of ATA used. Indeed, at 2.10^{-4}M, ATA reduces by only approximately 20 % the incorporation of ^{14}C-leucine into proteins. This is compatible with an inhibition of the initiation of protein synthesis. Figures 4a and 4b show respectively the ultracentrifu-

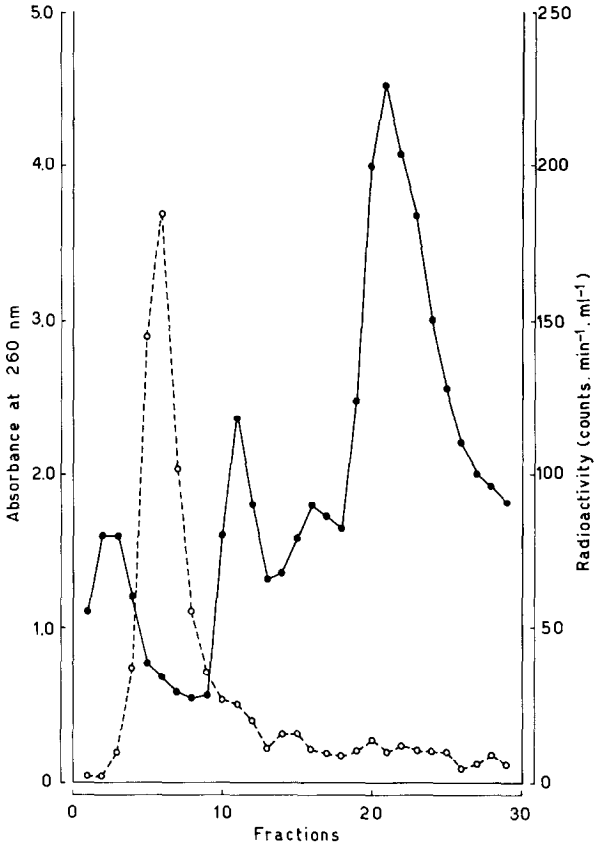

Fig. 3. Sedimentation profile in a sucrose gradient of ribosomes with ^{32}P labelled mRNP and 0.0002 M ATA. For details, see Materials and Methods.

o —— o Absorbance at 260 nm.
o --- o ^{32}P activity retained on nitrocellulose membrane filters.

gation patterns of ribosomes from such systems after two minutes of incubation with and without ATA 2.10^{-4}M. We see, by comparing Figures 4a and 4b that in the profile of the sample incubated with ATA, the shoulders on the heavy sides of each polyribosomal peak have disappeared. The shoulders are believed to correspond, for each polyribosome size, to an initiating entity, i.e. the polyribosome on which an additional 40S particle is attached at the initiation site of the messenger RNA (23).

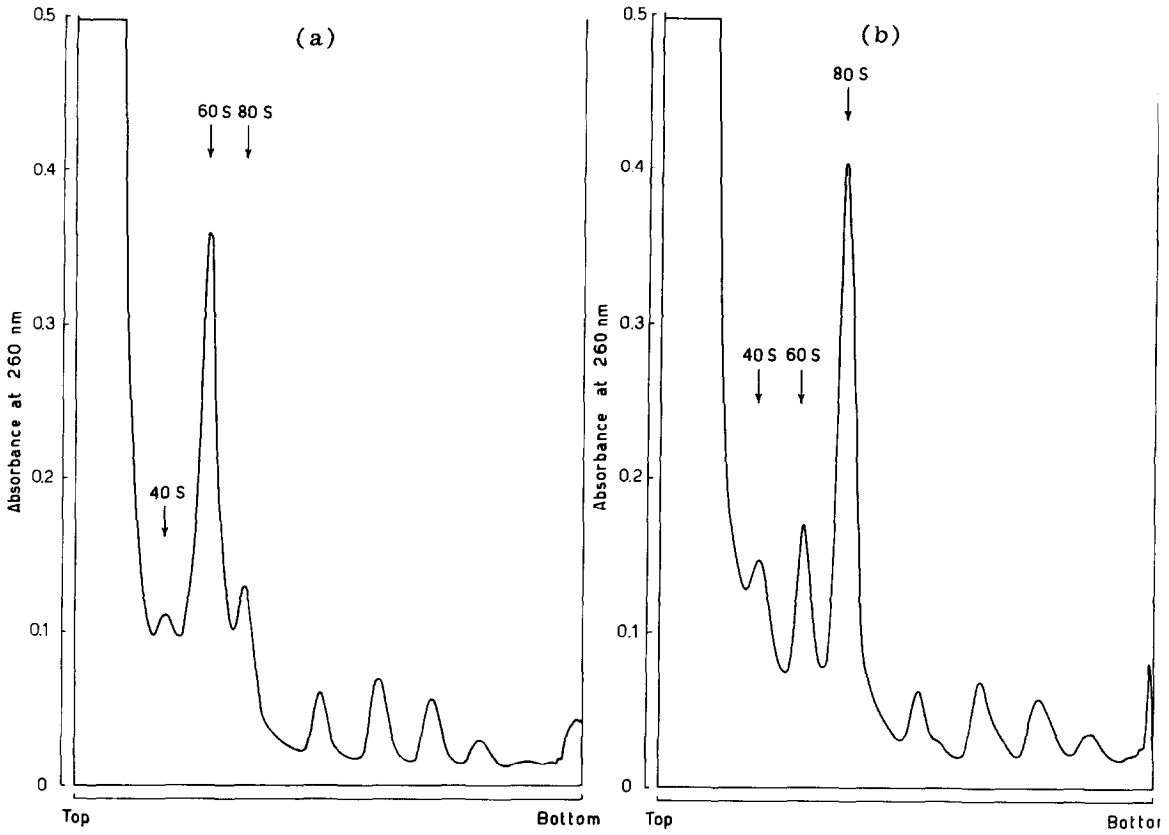

Fig. 4a Sedimentation profile in a sucrose gradient of a 200 µl aliquot of an "in vitro" protein synthesizing system incubated for two minutes in the presence of 0.0002 M ATA. For details, see Materials and Methods.

Fig. 4b Sedimentation profile in a sucrose gradient of 200 µl aliquot of a control "in vitro" protein synthesizing system incubated for two minutes. For details, see Material and Methods.

This confirms, in conditions under which the elongation of polypeptide chains remains unaffected, the results obtained by the binding test. On the other hand, Fig. 4a shows that ATA induces an important lowering of the sedimentation constant of 80S ribosomes which sediment now at 60S. Indeed, as the quantity of 40S subunits is not changed, the increase of the 60S peak cannot be due to the dissociation of the 80S ribosomes. This observation must be related to these made by Grollman et al. (15) who showed

that in E. coli also, ATA induces a modification in the sedimentation constant of ribosomes which, in this case, sediment more rapidly.

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