FLUORESCENCE STUDIES ON THE 30 S RIBOSOME ASSEMBLY PROCESS

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Received 21 September 1970

E. coli 30 S ribosomal subunits may be reconstituted *in vitro* from their protein constituents and 16 S RNA [1]. The complexity of the assembly process was clarified to a large extent by Mizushima and Nomura in a recent publication [2] where the authors proposed an assembly map for the 30 S ribosome.

We were also concerned with the assembly process and chose as experimental principle to introduce a fluorescent marker (ethidium bromide) in the reconstitution system and to follow the evolution of the fluorescence intensity during the reconstitution process. Our results indicate that some base-paired regions of the 16 S RNA molecule are involved in the assembly process and that the recognition of these regions by 30 S ribosomal proteins is highly specific. Moreover, it appears that only a few ribosomal proteins interact with the 16 S RNA base-paired regions and that an intermediate complex forms in the presence of ethidium bromide. This complex has a sedimentation coefficient of about 20 S and a protein composition similar to that one of the ribosomal subunit precursor 21 S accumulating in a cold sensitive mutant [3]. This provides strong correlation between *in vivo* and *in vitro* results and supports the relevance of *in vitro* assembly studies to the biological process.

16 S RNA, total, core and split proteins from 30 S ribosomes were prepared as described in Traub and Nomura paper [1]. Pure ribosomal proteins were isolated on carboxymethyl cellulose columns [4] and electrophoresis performed in 8 M urea gels, pH 4.4, acrylamide 7.5% and bisacrylamide 0.8%.

30 S ribosomes reconstitution was followed by the fluorescence variation of ethidium bromide charged 16 S RNA. As shown by Lepecq et al. [5], interaction of ETB with RNA involves two types of binding sites: a) strong sites corresponding to the intercalation of the dye into double-helical regions with as result a considerable enhancement of fluorescence; b) weak sites corresponding to cationic binding of ETB to phosphate groups without fluorescence stimulation. In our working conditions (0.37 M K^+, 2×10^{-2} M Mg^{2+}), ETB-phosphate binding certainly does not occur [5] at low dye concentration. (fig. 1)

The standard assay was performed as follows: ethidium bromide was added to 1 A_{260} nm 16 S RNA in 1 ml of the reconstitution buffer (described by Traub and Nomura [1] in order to obtain a 2.5×10^{-5} M final concentration. The fluorescence index of the mixture was recorded on a Jobin Yvon fluorimeter after stabilisation of the temperature (40°). 1.2 A_{260} nm equivalent of total 30 S proteins (TP30) were added to the 16 S/BET mixture and the fluorescence variation was recorded as a function of time.

Fig. 2 shows the chasing of ETB from its binding sites on 16 S RNA upon addition of TP30 proteins. Chasing is very rapid and appears completed in about 3 to 4 min. Successive additions of partial TP30 amounts results in the same chasing effect, indicating a definite stochiometry. Chasing of ETB from its binding sites on 16 S RNA corresponds to the recognition of particular RNA regions by TP30 proteins, as shown by the fact that the phenomenon is highly specific, occurring only with TP30 proteins and not when nucleohistones or 50 S ribosomal proteins are used. Moreover no displacement of ETB by TP30 proteins is observed from a 23 S RNA–ETB complex. It is interesting that

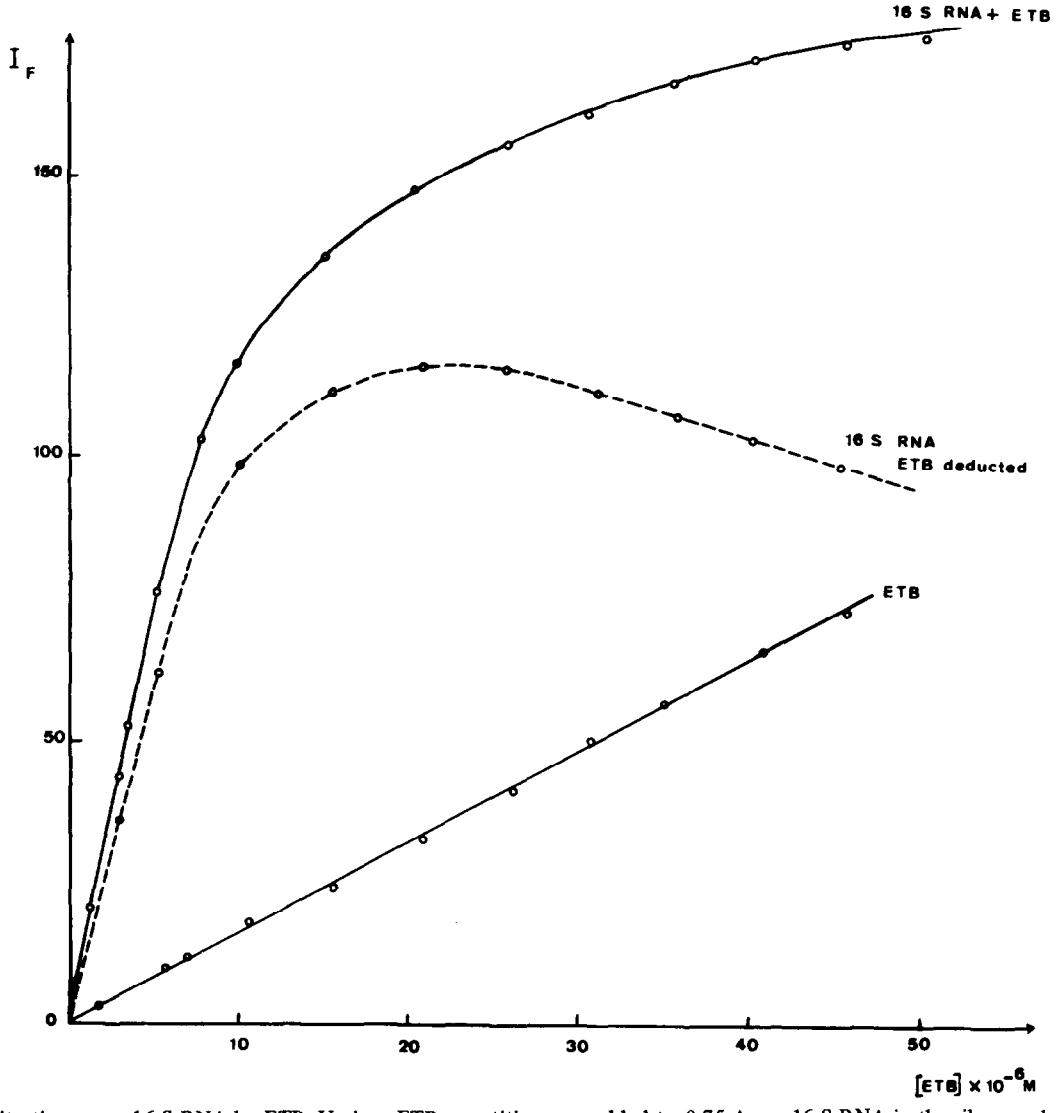

Fig. 1. Titration curve 16 S RNA by ETB. Various ETB quantities were added to 0.75 A_{260} 16 S RNA in the ribosomal reconstitution conditions (1) and the fluorescence index (I_f) measured with a Jobin Yvon fluorimeter.

the release of ETB is only partial, a large amount of the dye remaining fixed on 16 S RNA. The fluorescence level attained after a few minutes reaction is quite higher than the one we observe with 30 S ribosomes reconstituted without ETB (see fig. 2). This suggests that the assembly process is in some way impeded by ethidium bromide and that an intermediary complex is formed between 16 S RNA and a few particular ribosomal proteins. We would anticipate that these proteins have the highest affinity for the 16 S RNA molecule.

The first step towards identification of the proteins involved in the intermediary complex was to check the effect of adding split and core proteins respectively. Table 1 reveals that the core proteins (CP30) give a near maximum chasing effect whereas split (SP30) contribute only slightly, their effect being possibly accounted for by contaminating CP30 proteins.

Several pure 30 S ribosomal proteins added in stochiometric amounts were tested for ETB chasing and the results are summarized in table 2. It appears that proteins P14, P5, P4b and P4a displace ethidium bromide from the 16 S RNA molecule to a low but significant extent.

We intend to correlate these results with those of Mizushima and Nomura who found P4a, P4b, P5 and P14 among the six proteins which bind to the 16 S RNA [2] in partial reconstitution experiments.

In order to further identify the proteins fixed on the 16 S RNA in presence of ETB, we isolated the complex by centrifugation (16 hr at 40,000 rpm). The pellet was dissolved in buffer (tris 10^{-2} M pH 7.6, MgAc 1.4×10^{-2} M, NH_4Cl 6×10^{-2} M and mercaptoethanol 6×10^{-3} M), an aliquot was taken to estimate the sedimentation coefficient of the complex, the remaining sample being digested

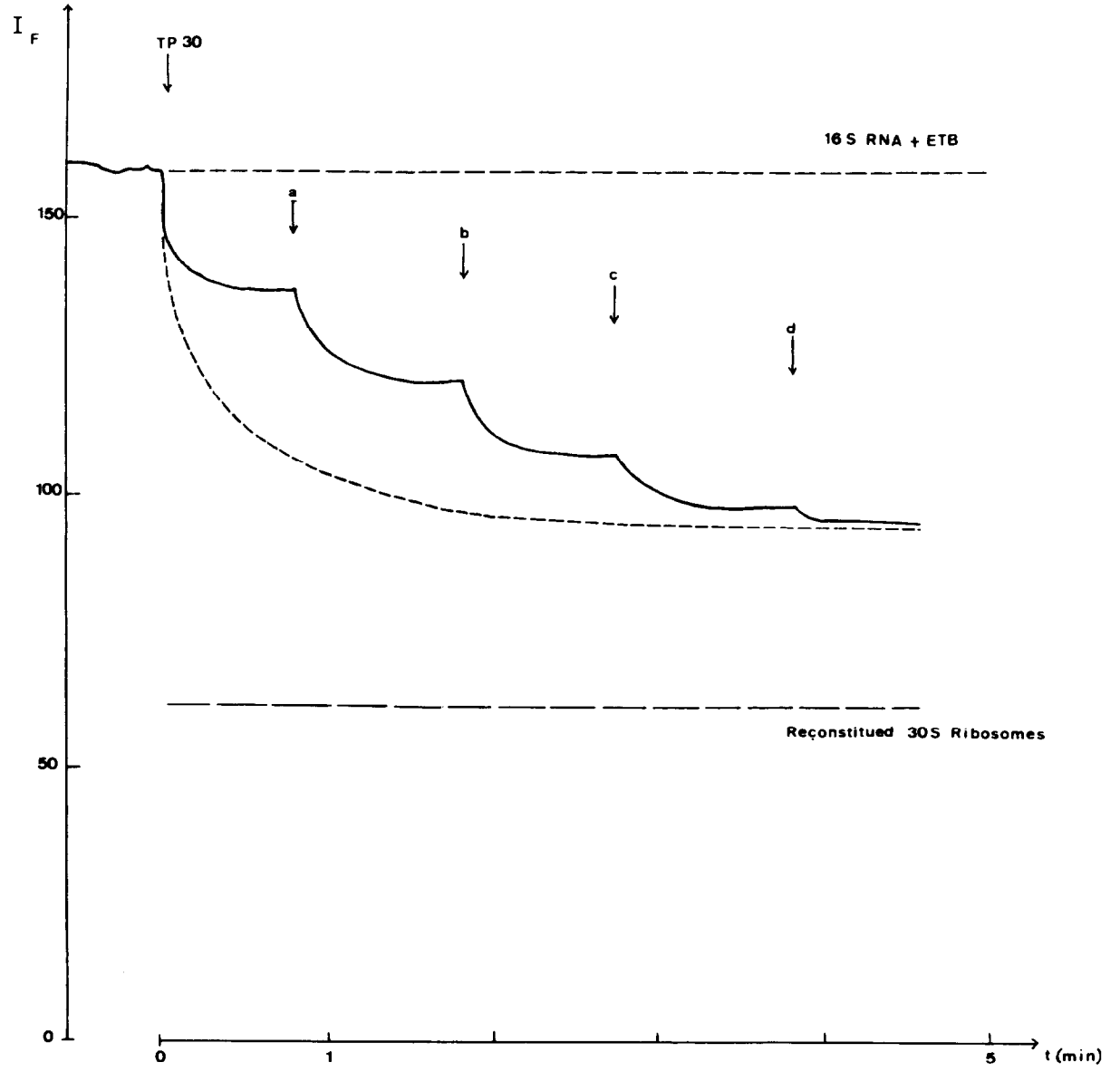

Fig. 2. ETB chasing by TP30 proteins: - - - 1.2 Absorbance equivalent; —— a, b, c, d partial amounts.

Table 1
ETB chasing by split and core proteins

Additions	Fluorescence index		
	Initial	Final	% Chasing
TP30	157	92	41.4
Σ Pi	160	95	40.0
$SP30_{(rec)}$	182	168	7.7
$CP30_{(rec)}$	120	75	37.5
$SP30_{(CsCl)}$	190	173	9.0
$CP30_{(CsCl)}$	180	125	36.1

Σ Pi stands for the complete mixture of isolated proteins $SP30_{(rec)}$ and $CP30_{(rec)}$ for the mixture of respectively the isolated split and the isolated core proteins, and $SP30_{(CsCl)}$ and $CP30_{(CsCl)}$ for split and core proteins isolated on cesium chloride gradient.

with pancreatic and T1 RNAases. Proteins were analysed on polyacrylamide gels and compared on split gels to specific isolated proteins for identification. Fig. 4 shows the sedimentation profile of the complex, the sedimentation coefficient of which is estimated at 20 S, with reference to 30 S and 50 S labelled markers. ETB charged 16 S RNA can be seen as a shoulder of the main peak.

Polyacrylamide gel analysis of the complex (fig. 3) reveals eight bands corresponding to the P4a, P4b, P5, P8, P10, P13 and P14 proteins and to one or both of the P9 and P10a proteins (proteins P9 and P10a overlapped in our bands). The proteins compositions of the 20 S particle was found to be very close to that of the

Table 2
Effect of pure proteins on the chasing of ETB.

Addition	Fluorescence index		
	Initial	Final	% Chasing
P15	195	190	2.5
P14	193	185	4.1
P10	192	191	0.5
P6	195	191	2.
P5	190	175	7.5
P4b	182	168	7.7
P4a	185	175	5.4
P3a	182	178	2.2

The protein nomenclature of Nomura et al. [6] is used.

Table 3
Protein composition.

20 S ETB particle	21 S Cold sensitive mutant
P4a	P4a
P4b	P4b
P5	P5
P8	P8
P10	–
P9 or/and P10a	P9 and P10a
P13	P13
P14	P14
	P3a or P3b or P3c

21 S ribosomal precursor accumulating in some cold sensitive mutants [3] (table 3).

Our results strongly suggest that a particular class of proteins directly recognize double helical regions of the 16 S ribosomal RNA. Further investigations are however needed to obtain a precise mechanism of ETB release. This may result either from partial unwinding or some double stranded regions or from a definite stereochemical readjustment of some double

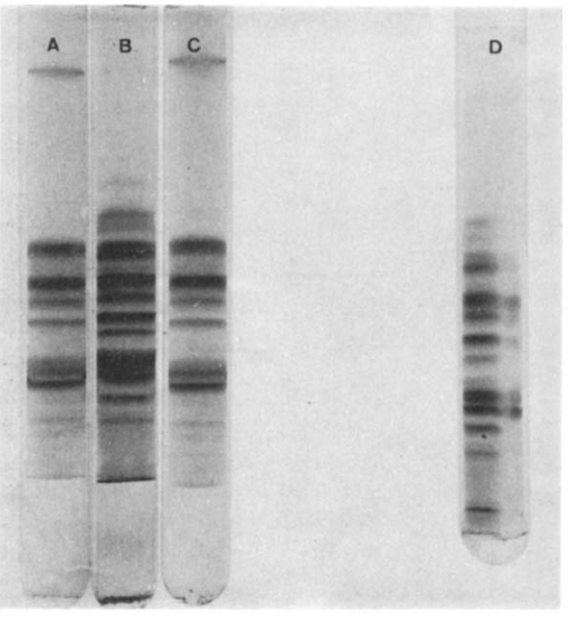

Fig. 3. (A) and (C): protein composition of the 20 S particle; (B): protein composition of the 30 S ribosome; (D): split gel of A and B.

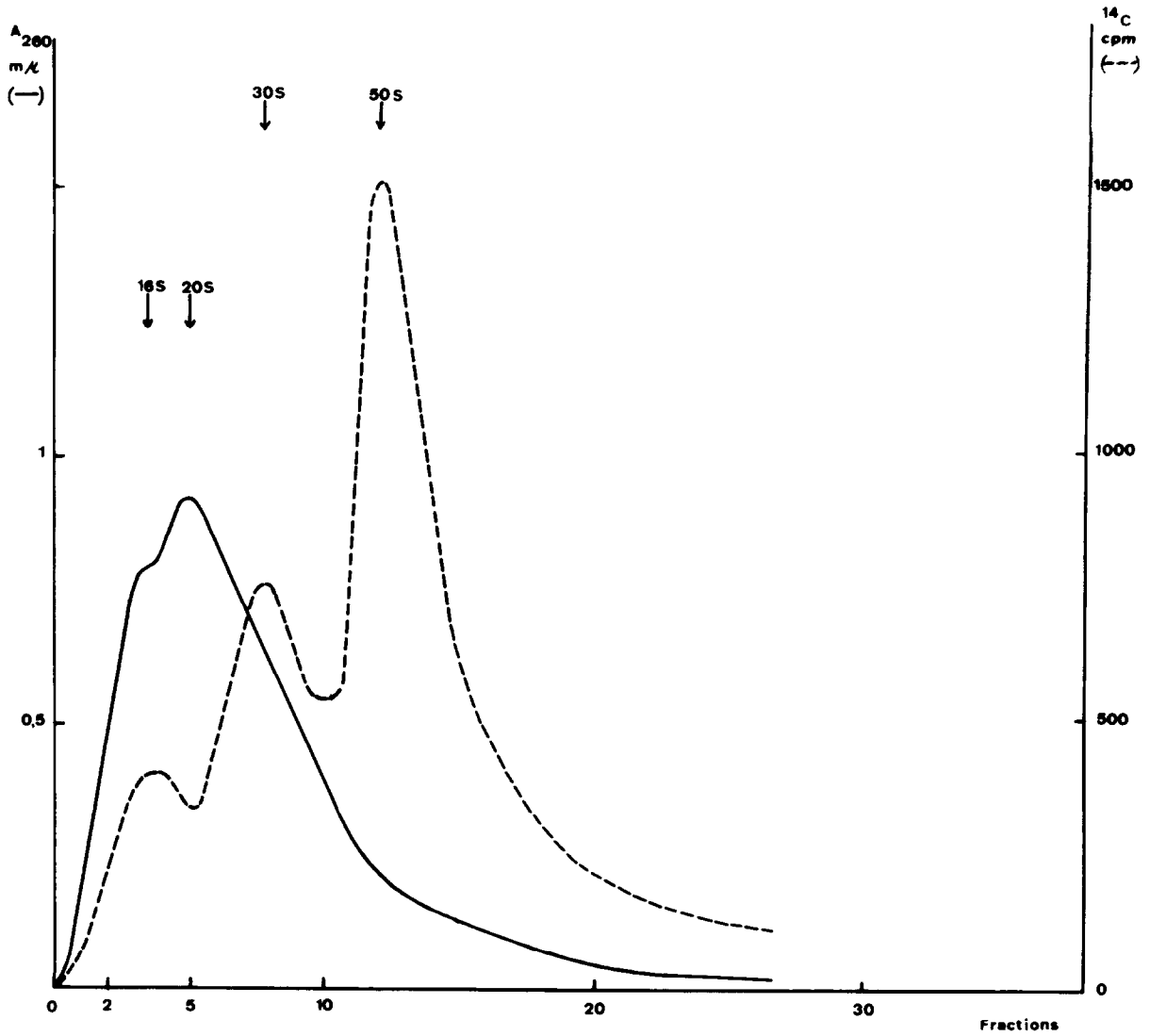

Fig. 4. 10%–30% saccharose gradient centrifugation of the intermediary complex, 2½ hours at 39,000 rpm in a SW 65 Ti rotor.

helical region(s) in the immediate vicinity of the protein. The blocking of the 30 S ribosome reconstitution at the 20 S step might result from the inability of some proteins to displace ETB and/or from a conformational change of the 20 S particle by ETB which would prevent completion of the structure.

Acknowledgements

This work was supported, in Belgium, by Euratom (contract 007–61–10 ABIB) and the Fonds de la Recherche Fondamentale Collective and, in France, by the Fonds de Développement de la Recherche Scientifique et Technique, du Centre National de la Recherche Scientifique, du Commissariat à l'Energie Atomique, de la Ligue National Française contre le

Cancer et de la Fondation pour la Recherche Médical. A. Bollen is Chargé de Recherches in the Fonds National de la Recherche Scientifique of Belgium.

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