Volume 232, number 1, 29–34 FEB 05814 May 1988

Topography and stoichiometry of acidic phosphoproteins in rat liver 60 S ribosomal subunit

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Received 18 February 1988

In reconstitution experiments of active 60 S subunits from inactive core particles obtained by using dimethyl maleic anhydride (DMMA), we observed that the phosphoproteins P1-P2 were extracted from the subunit by DMMA as a complex with other proteins. This complex was separated by electrophoresis and zonal centrifugation and shown, after ^{125}I iodination of its components, to contain L22 and S12 in addition to P1-P2. Results suggest that it contains two copies of P1-P2 for one of L22 and S12.

Ribosome; 60 S subunit; Phosphoprotein; Reconstitution

1. INTRODUCTION

The strongly acidic proteins of the large ribosomal subunit are probably among the most interesting components of the ribosome structure. In *Escherichia coli*, these proteins (L7/L12), in fact a unique polypeptide with its amino terminal either free (L12) or blocked (L7), are present as a pair of dimers, each of which is anchored to the ribosome through interaction with a common protein L10. The N-terminal part of L7/L12 is essential for the dimer formation and the binding to L10. They form the stalk extending away from the large subunit. The four copies of L7/L12 are easily and selectively removed from the ribosome using 50% ethanol/1.0 M NH_4Cl and can be reassociated. They are involved, most likely their C-terminal part, in the GTP-dependent reactions of protein synthesis, in particular translocation. L7/L12 probably induce the appropriate particle conformation for the interaction with elongation factors to take place under the proper conditions (see [1]).

The large subunits of eukaryotic ribosomes also contain strongly acidic proteins (named P1-P2 in rat liver) that are structurally related to L7/L12. These proteins exist in phosphorylated states and are exchangeable with cytoplasmic counterparts [2–4]. Some attempts have been made to determine their stoichiometry and spatial arrangement. An average of 3.2 copies of proteins P per ribosome has been reported in *Saccharomyces cerevisiae* [2]. In *Artemia salina* 60 S subunits, an earlier study indicated the presence of 1.8 copies of P2 and 0.9 copy of P1 [5]. More recently, within the same subunits, two homodimers: P1-P1 and P2-P2 were found to be anchored to an almost unknown protein: A33, called protein Po by other investigators [6]. These results are consistent with two other data indicating a strong affinity between P1-P2 and A33 to form a 140 kDa complex in *A. salina* ribosomes, and a close proximity of P1 and A33 in rat liver ribosomes [4,7]. Then, it has been suggested that, in eukaryotes, protein A33 might play the same role as protein L10 in *E. coli* and form a pentameric complex with the two dimers $(P1)_2$ and $(P2)_2$. However, further studies are

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00145793/88/$3.50

necessary to confirm this assertion, particularly in the case of mammalian ribosomes. Here, due to their particular properties, P1-P2 and A33 are difficult to identify (see section 3). Moreover procedures for the selective extraction of P1-P2 are known (ethanol/KCl), but not for A33. The role of mammalian P proteins in the interaction of elongation factors with ribosomes has been proved in three reconstitution experiments, including ours [8–10]. The two earlier ones used total rat liver 80 S ribosomes specifically deprived of P1-P2 by ethanol-KCl washing. We used purified 60 S subunits and demonstrated that addition of purified P1-P2 to inactive residual core particles lacking specifically proteins P1-P2, A33, X, L10a, L12 and L22 can reconstitute 60 S subunits which have recovered part of their EF-2-dependent GTPase and polyphenylalanine synthesizing activities. The split proteins were removed after treatment of 60 S subunits with 2,3-dimethylmaleic anhydride (DMMA), a reagent for proteins which at pH 8.2 substitutes a negatively charged residue for each amino group and is easily removed at pH 6.0. Within the DMMA wash, active in reconstitution of subunits, P1-P2 were most probably complexed to other proteins for they could not be inactivated by phosphatase as when they were purified [11]. Here we identified and quantified the protein components of this complex that is released during DMMA treatment of 60 S subunits.

2. MATERIALS AND METHODS

2.1. *Materials*

Rat liver 60 S ribosomal subunits were prepared by zonal centrifugation as described using free polysomes [12]. DMMA was purchased from Sigma, ^{125}I from Amersham.

2.2. *Two-dimensional gel electrophoresis of DMMA-treated subunits*

5 A_{260} units of 60 S ribosomal subunits in 50 mM K^+-Hepes, pH 8.2, 25 mM KCl, 1.5 mM $MgCl_2$, 20 mM 2-mercaptoethanol were treated with DMMA at a reagent molar excess of 15000, as described in [10]. The preparation was analyzed on two-dimensional gel electrophoresis as previously described [13]. The first dimension was performed under non-denaturing conditions using a 2.4–7.5% polyacrylamide concentration gradient in 36 mM Tris/acetate, pH 7.8, 30 mM K phosphate, 1 mM Mg acetate; the second-dimension gel in 12% polyacrylamide with 0.2% SDS and 6 M urea, in order to resolve the protein components of the complex released by DMMA. For identification every protein spot of the complex was excised from the slabs, stained with Coomassie blue and labeled with ^{125}I using the chloramine T procedure [14]. The labeled proteins were extracted twice with 1% SDS, 0.1 M phosphate buffer, pH 7.0, at 37°C for 3 h, mixed with 300 μg of 60 S ribosomal proteins as a carrier and then recovered by precipitation with 8 vols acetone, 0.1 M HCl. The proteins in 6 M urea were reprecipitated 3 times by acetone as described above and subjected to two-dimensional polyacrylamide gel electrophoresis in an acidic-SDS system [15]. The gels stained with Coomassie blue and dried were autoradiographed according to [16]. Several autoradiograms of different exposure times were made for each slab. The darkness of the protein spots was quantified by direct scanning of faintly exposed films using a Vernon photometer equipped with an integrator. It was linearly related to the exposure time and to the number of radioactive disintegrations, in agreement with [16]. Deviation of the protein quantification data obtained from different autoradiograms was ± 10%. This method of labeled protein quantification by direct scanning of autoradiograms was preferred to the direct counting of the labeled proteins because of the difficulty in excising reference protein L22 free from protein S12 (fig.2).

3. RESULTS AND DISCUSSION

In order to obtain direct evidence that some of the proteins released by DMMA were associated to form a complex (see section 1), DMMA-treated 60 S subunits were submitted to a two-dimensional gel electrophoresis, using non-denaturing conditions in the first dimension and SDS-urea in the second. The spots of proteins were visualized by staining the gel with Coomassie blue. Fig.1 shows a typical electrophoretic analysis. The proteins seen at the left of the electrophoretogram originated from the residual DMMA-core particles that could not migrate in the first-dimension gel. Consistently, whatever the migration time used in the first dimension (1.5–2.5 h), 5 proteins migrated together in this first dimension as a complex and were resolved as faintly stained spots in the second dimension (spots I–V). Under the electrophoretical conditions used no material migrated in the gel plates when using either control 60 S subunits or total free 60 S proteins treated or not with DMMA.

As the next step each protein spot (I–V) was cut out, labeled with ^{125}I and the protein(s) identified by two-dimensional gel electrophoresis (fig.2). The results of several autoradiograms are summarized in table 1 with the quantification of labeled proteins by direct scanning of the radioiodinated spots (see section 2). It appears that the observed molecular mass of spots I–V agree with those of the proteins they contain except for spot II. This

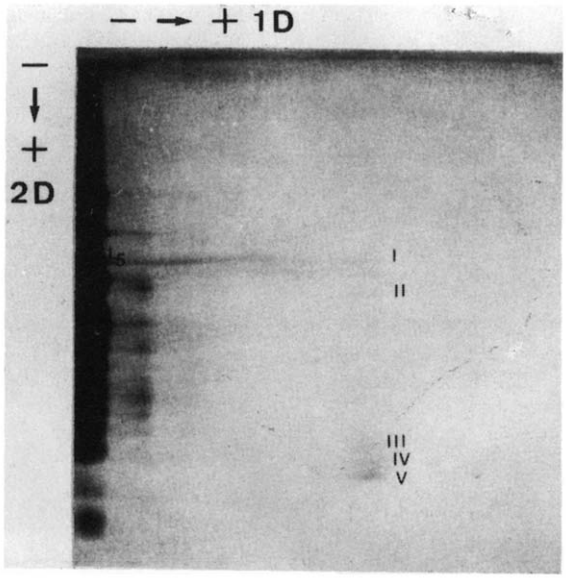

Fig.1. Two-dimensional polyacrylamide gel electrophoresis of DMMA-treated 60 S subunits. 60 S subunits treated with DMMA at a 1:15000 mol/mol ratio (1 h at 20°C) were electrophoresed as described in section 2. The spots of protein were stained with Coomassie brilliant blue.

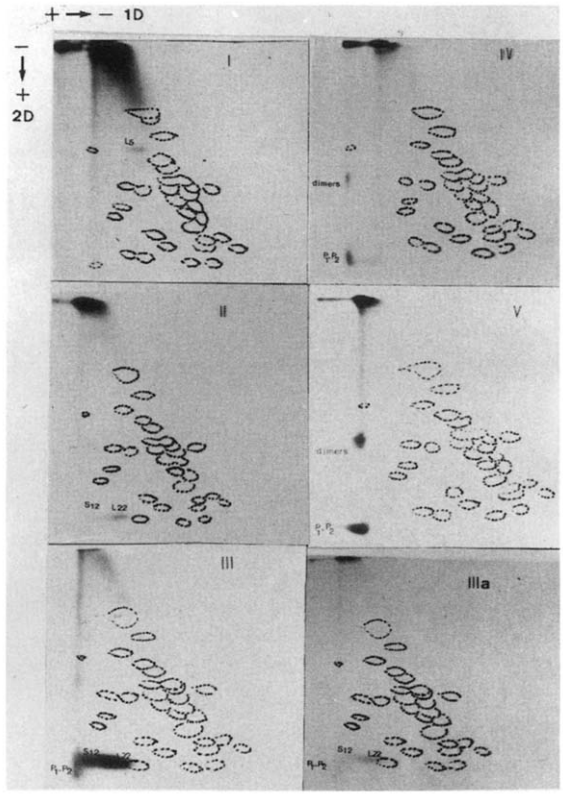

Fig.2. Identification of proteins from the complex released by DMMA. Protein spots (I–V) from the gel plate seen in fig.1 were radioiodinated and submitted to two-dimensional (acidic-SDS) gel electrophoresis with carrier total 60 S proteins (slabs I to V, respectively) as described in section 2. The gel slabs were stained with Coomassie blue, dried and autoradiographed 27 h. A 5 h autoradiograph of slab III is also given (IIIa). The positions of stained spots of carrier 60 S proteins are indicated by dotted lines on the autoradiographs. The position of protein A33 (or P0) which was not identified in [15], corresponds to the dotted spot located above P1-P2 dimers.

protein spot of relatively high molecular mass (29 kDa) contains two very low molecular mass proteins: L22 and S12 whose molecular mass sum (31 kDa) is close to its own. This indicates the presence within spot II of an L22-S12 protein complex. Most of this complex dissociated during electrophoresis in the second dimension for 87% of total L22 plus S12, measured from the darkness of the radioiodinated spots, was recovered as free molecules in spot III. The presence of phosphoacidic proteins within spot III might result from a contamination by the neighbouring spot IV. Radioiodinated phosphoacidic proteins P1 and P2 could not be identified unambiguously one from the other in fig.2 for they were almost never seen as two distinct spots on the gels of carrier 60 S proteins used for their identification. Consequently, these proteins are referred to in the text as P1-P2 although they seem to be well separated in fig.1: the molecular masses of spots IV and V would agree with those of P1 and P2, respectively. Results from fig.2 and table 1 show that about half of P1-P2 from both spots, IV and V, dimerized when they were radioiodinated, freed of DMMA by acidification (during precipitation by acetone-HCl, see section 2) and identified by two-dimensional gel electrophoresis in an acidic-SDS system. Staining gel plates identical to that of fig.1 with silver for RNA analyses [17] indicated that electrophoresis of DMMA-treated subunits only released traces of 5 S and 5.8 S RNA, almost exclusively as free molecules, in agreement with the RNA content of residual DMMA core-particles, which was apparently not modified (not shown, see also [10]).

In other experiments the low molecular-mass material released from DMMA-treated 60 S

Table 1

Protein composition of the complex isolated from DMMA-treated 60 S ribosomal subunits

Protein spots[a]		Proteins identified within the spots				Proposed composition of the complex		
Number	Observed M_r[b]	Number	M_r[c]	Composition[c] (number of Tyr residues)	Darkness on autoradiograms[d]	Proteins	Darkness/ number of Tyr	mol proteins/ mol of 60 S subunit
I	36000	L5	32500	9	115	L22	181	1.00
II	29000	L22	16100	4	100	P1-P2	349	1.93
		S12	14900	3	55	S12	159	0.88
III	17000	L22	16100	4	625	L5	13	0.07
		S12	14900	3	421			
		P1	16100	1	259 (monomers)			
		P2	15200	2				
IV	16000	P1	16100	1	113 (dimers)			
		P2	15200	2	157 (monomers)			
V	15000	P1	16100	1	257 (dimers)			
		P2	15200	2	260 (monomers)			

[a] Protein spots were cut out from the two dimensional gel seen in fig.1

[b] M_r values of the proteins were determined using bovine serum albumin (67000), aldolase (40000), deoxyribonuclease (31000), trypsin (23900), ribonuclease (13700) and cytochrome *c* (12500) as markers

[c] M_r values and Tyr composition of the proteins are taken from [27–29]

[d] Darkness of the radioiodinated spots was measured on the 27 h exposed autoradiograms (see section 2)

subunits was purified by centrifugation through a 15–50% sucrose gradient (fig.3). The tracing at 280 nm revealed a prominent 4–5 S peak (I) whose protein content was analyzed using the two-dimensional polyacrylamide gel system described in fig.1. This peak was found to contain the same protein complex as that seen in fig.1. This strongly suggests that the complex, P1-P2-L22-S12, resistant to DMMA action, represents a protein neighbourhood in the functional 60 S subunits, that is maintained by groups not reactive towards DMMA. The fact that P1-P2 and L22 were located near 5 S RNA and protein L5 would recall that, in *E. coli*, L7/L12 are close to protein ECL5 that binds to 5 S RNA [18,19].

Our experiments show that DMMA extracted P1-P2 and L22 as a complex and we know that this extraction was complete [10]. There should have been no loss of these proteins during the purification and analyses of the complex by two-dimensional gel electrophoresis since the treated subunits were directly put on the gel (fig.1). Hence from this experiment and the corresponding autoradiographs (fig.2) we attempted to determine the number of copies of P1-P2 in comparison with protein L22 that we assumed to be present in one copy, making the following hypotheses: the chloramine T method almost exclusively labeled Tyr residues [20]; every protein from spots I–V, separated under denaturing conditions, had all its Tyr residues labeled; the darkness observed at the origin of the autoradiographs was due to artefactual material other than ribosomal proteins, that was labeled within the gel at the same time as the protein to be identified. [Clearly the large amount of radioactivity that trailed from the origin in fig.2 (I) was not due to protein L5, because this protein remained for the most part in the core particle (see fig.1, left and [10]). On the other hand proteins would be solubilized in the second dimension electrophoresis performed in the presence of SDS-urea.] The number of copies of each protein present within the complex was deduced from the value of the ratio: darkness of the radioiodinated protein on the autoradiogram/number of Tyr residues, relative to the same ratio determined for protein L22, under the same conditions. Our results indicate that the complex contained two copies of (P1-P2) per mol of L22 with traces of L5. The presence of 0.9 copy of S12 means that this protein was stripped with 60 S subunits from the 40 S ones during the dissociation of the two

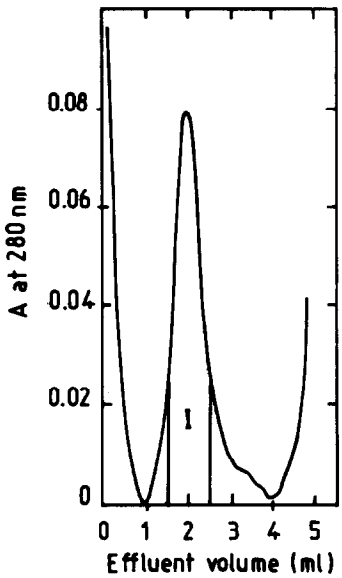

Fig.3. Rate-zonal sedimentation of the low molecular-mass material released by DMMA. 20 A_{260} units of DMMA-treated subunits were applied to a 15–50% linear sucrose density gradient in 50 mM K^+ Hepes, pH 7.4, 25 mM KCl, 1.5 mM $MgCl_2$, 20 mM 2-mercaptoethanol. Centrifugation was at 4°C for 22 h at 59000 rpm using a Beckman SW60 rotor. The absorbing material seen at the top of the gradient corresponds to DMMA (left).

subunits. The presence of protein S12 on the 60 S subunits has already been reported [21]. This acidic protein, like P1-P2, contains a large number of hydrophobic amino acids [22].

Available data on the properties of protein L22 that was found complexed with P1-P2 indicate that it is an external protein that, like P1-P2, associates to ribosomal particles at later stages of the maturation process [23]. It does not seem essential for the functioning of 60 S ribosomal subunits because we recently reconstituted active subunits using 50% ethanol/0.5 M KCl protein extract and DMMA-core particles, both lacking L22 (Lavergne, J.P. et al., unpublished). The same holds true for protein A33 that was reported to be associated to $(P1)_2$, $(P2)_2$ in *A. salina* ribosomes [6] possibly with other proteins (the reported molecular mass of the complex, 140 kDa, is much higher than that determined from the stoichiometry and molecular masses of P1, P2, and A33 [4]). However, we cannot exclude the possibility that protein L22, with or without A33 (see below), is needed to attach P1-P2 to the core particles. In *E. coli*, the absence of protein L10, in reconstitution experiments using only residual CsCl core particles and L7/L12, can be circumvented by an excess of the latter [24]. In our recent reconstitution experiments, we found that a large excess of an ethanol-KCl extract of 60 S subunits, containing P1-P2, was needed to reactivate the DMMA core particles. We did not find A33 in the complex present in the DMMA wash, although we showed that this protein was present in this wash [10]. However we cannot discard the possibility that A33 had escaped our investigation during the 2 steps of identification of the protein complex (figs 1 and 2). A33 is known to be hardly stained by Coomassie blue, to have a limited solubility and to fail to enter the first-dimension gel (in the acidic-SDS system). Other attempts to identify proteins A33 and P1, P2 within our complex using, as described [4,25,26], the sera of patients with systemic lupus erythematosus (SLE) that reacted against 60 S proteins having molecular masses of 38, 19 and 17 kDa (determined by one-dimensional gel electrophoresis) were unsuccessful. In fact among these particular sera, which represented only a very small part of the SLE sera that reacted against ribosomal proteins, only one (not obtained in sufficient amounts for our study) among one hundred was shown, by two-dimensional electrophoresis and immunoblotting, to react only with A33 and P1, P2. The others reacted with additional proteins of M_r close to that of A33 and could not serve for A33 specific identification (Absi, M. et al., unpublished).

Acknowledgements: This work was supported by the 'Institut National de la Santé et de la Recherche Médicale' (grant 862020), the 'Centre National de la Recherche Scientifique' (UM 24) and the 'Université Lyon I'.

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