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Role of acidic phosphoproteins in the partial reconstitution of the active 60 S ribosomal subunit

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We have recently shown that rat liver 60 S ribosomal subunits active in protein synthesis can be reconstituted from inactive core particles lacking 30% of the total proteins, mainly L10a, L12, L22, L24, A33 and the acidic phosphoproteins P1–P2, obtained by treatment of 60 S subunits with dimethylmaleic anhydride [(1987) Eur. J. Biochem. 163, 15–20]. In this study, an ethanol extract of the 60 S subunit which contains only P1–P2 was also shown to be effective in reconstitution with the DMMA-core-particles: it strongly stimulated the EF-2-dependent GTP hydrolysis and, to a lesser extent, polyphenylalanine synthesis; like the DMMA wash it shifted the thermal denaturation curve of the DMMA-core particles towards that of control subunits. Prior dephosphorylation of the ethanol extract by alkaline phosphatase inhibited the reconstruction process.

Ribosome; 60 S subunit; Phosphoprotein; Reconstitution

1. INTRODUCTION

Since the detection of acidic proteins L7–L12 in the large subunit of *E. coli* ribosomes and their associated activities in translocation, GTP hydrolysis and binding of several factors (initiation factors IF-2, elongation factors EF-G and EF-Tu, releasing factors RF-1 and RF-2), several studies have been made on eukaryotic proteins analogous to L7–L12. Such proteins, in most cases phosphorylated, have been found in all eukaryotic ribosomes (see [1]). In particular, the rat liver 60 S ribosomal subunit contains multiphosphorylated acidic proteins P1–P2 which are extracted selectively in the presence of 50% ethanol at low salt concentration [2]. More recently, it has been reported that P1–P2 removed from 80 S rat liver ribosomes by ethanol under these conditions can be added back to the corresponding core particles to produce partially reactivated ribosomes in both polyphenylalanine synthesis and EF-2-dependent GTPase reactions. Dephosphorylation of P1–P2 caused a decrease in the ability of these proteins to reactivate the polyphenylalanine synthesis ability of the core ribosomes. However, attempts to extract the 60 S rat liver ribosomal subunits by ethanol resulted in particles which could not be reactivated [3].

We have recently reported that rat liver 60 S ribosomal subunits active in EF-2-dependent GTPase and polyphenylalanine synthesizing activities can be reconstituted from inactive core particles, deprived of specific proteins among which are the acidic phosphoproteins P1–P2, using a reagent for protein amino groups dimethylmaleic anhydride (DMMA) [4]. This reagent, which at pH 8.2 substitutes a negatively charged residue for each amino group and is easily removed at pH 6.0, had been previously shown to be able to dissociate

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proteins from yeast 60 S ribosomal subunits reversibly [5,6]. The present report concerns the effect of proteins P1–P2, obtained from rat liver 60 S subunits by alcohol extraction at low salt concentration, on the reactivation and the conformation of the residual DMMA core particles. The influence of covalent phosphate on the function of P1–P2 has also been studied.

2. MATERIALS AND METHODS

2.1. *Materials*

60 S ribosomal subunits were prepared by zonal centrifugation as described [7] using free polysomes. 95% pure EF-2 was prepared from rat liver according to [8]. Agarose-immobilized alkaline phosphatase and DMMA were purchased from Sigma. L-[^{14}C]Phenylalanine (12 Bq/pmol) and [γ-^{32}P]GTP (962–1110 Bq/pmol) were obtained from the 'Commissariat à l'Energie atomique' and from Amersham (England).

2.2. *Treatment of the ribosomal subunits with DMMA*

60 S ribosomal subunits in 50 mM Hepes (K^+), 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 [4]. The preparation was centrifuged over a 10% sucrose cushion in the same buffer. The upper layer of the supernatant containing the split proteins and the sediment were dialyzed separately against 20 mM sodium-cacodylate, pH 6.0, 25 mM KCl, 1.5 mM $MgCl_2$, 5 mM 2-mercaptoethanol (buffer A) in order to regenerate the modified amino groups.

2.3. *Extraction of P1–P2 and treatment with phosphatase*

Proteins P1–P2 were selectively extracted from 60 S subunits (40 A_{260} units), suspended in 50 mM triethanolamine-HCl, pH 7.4, 80 mM KCl, 5 mM $MgCl_2$, 20 mM 2-mercaptoethanol by treatment with 1 vol. ethanol at 4°C, as described [2]. The ethanol extract was dialyzed against buffer A (16 h) and fractionated into 3 aliquots: a, b and c. Control sample (a) was kept in buffer A. Samples b and c dialyzed against 20 mM Tris-HCl, pH 7.8, 25 mM KCl, 1.5 mM $MgCl_2$, 5 mM 2-mercaptoethanol (3 h) were incubated (b) or not (c) with an excess of agarose-immobilized alkaline phosphatase (13 U, 25°C/40 min). Sample b was then freed of the agarose beads by centrifugation, and with its control (sample c) dialyzed against buffer A. Samples a, b and c were then used in reconstitution experiments. Assays of activity have shown that sample c behaves identically to sample a in reconstitution experiments.

2.4. *Reconstitution of active particles*

Reconstitution was conducted in two different ways. In each case, controls of DMMA-untreated 60 S subunits and DMMA-core particles were subjected to the same treatment as the reconstituted subunits. Initially, we used a published method of reconstitution (Method I [4]) in which mixtures of core particles plus split proteins (either twice the complementary amount of the DMMA wash, or 6 times the complementary amount of the ethanol extract, treated or not with alkaline phosphatase) were dialyzed against 20 mM Tris-HCl, pH 7.4, 300 mM KCl, 15 mM $MgCl_2$, 1.0 mM dithiothreitol, heated for 1 h at 37°C and then centrifuged through 10% sucrose cushions in 50 mM Tris-HCl, pH 7.4, 80 mM KCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol in order to remove unincorporated proteins from the reconstituted material (3.25 h at 4°C at 50000 rpm in a Beckman SW 50 rotor). We later developed another procedure (Method II) which was very fast and gave a better reconstitution when tested by activity measurements than the former. This procedure consisted in adding one quarter volume of 100 mM Hepes, pH 8.2, 1.5 M KCl, 75 mM $MgCl_2$, 30 mM 2-mercaptoethanol to the mixtures containing the core particles and the split proteins and in incubating the suspensions for 1 h at 37°C. The subunits reconstituted in this way could be used directly in polyphenylalanine synthesis assay but necessitated a dialysis against 20 mM Tris-HCl, pH 7.4, 30 mM KCl, 8 mM $MgCl_2$, 5 mM 2-mercaptoethanol prior to testing in the EF-2-dependent GTPase activity.

2.5. *Assays of activity*

Poly(U)-directed polyphenylalanine synthesis and EF-2-dependent GTPase assays were carried out as described [4] using limiting amounts of 60 S subunits or particles derived from them.

2.6. *Polyacrylamide gel electrophoresis*

Protein samples from the control subunits, the DMMA particles and the DMMA wash, were obtained as described [4] using the Mg^{2+}/acetic acid procedure [9]. Two-dimensional polyacrylamide gel electrophoresis using the acidic-SDS system [10] confirmed that the DMMA wash contained, among other proteins, the acidic phosphoproteins P1–P2, and that the DMMA residual particles were completely depleted of these proteins. It also confirmed that P1–P2 were the only proteins detectable in the ethanol extract prepared at low ionic strength [2] even when a very sensitive silver staining method was used.

3. RESULTS AND DISCUSSION

As can be observed in a typical experiment reported in table 1, the DMMA residual core particles showed only 13 and 6% of the original activities of 60 S subunits in poly(U)-directed polyphenylalanine synthesis and EF-2-dependent GTP hydrolysis, respectively. After addition of the DMMA split proteins, which contained P1–P2, among other proteins [4], polyphenylalanine synthesis activity was increased by a factor of 7 (nearly up to the control value) and EF-2-dependent GTPase activity by a factor of 9.5 (up to about two thirds of the control value), respectively. The ethanol split-protein fraction which contained P1–P2 alone [2] and was without any influence on

Table 1

Activities of particles reconstituted from DMMA cores and either DMMA-split or ethanol-extracted proteins

Preparations	Additions			Poly(Phe)-synthesis (%)	GTP-hydrolysis (%)
	Proteins		40S		
	DMMA wash	Ethanol extract			
Control	–	–	–	21	100
Control	–	–	+	100	
Core	–	–	–	5	6
Core	–	–	+	13	
Core	+	–	–	7	57
Core	+	–	+	88	
Core	–	+	–	5	44
Core	–	+	+	41	
Core	–	phosphatase treated	–	4	7
Core	–	idem	+	17	
Core	phosphatase treated	–	–	4	51
Core	idem	–	+	83	

Reconstitution was carried out according to Method II using either DMMA wash or ethanol extract treated or not with alkaline phosphatase (see section 2). Polyphenylalanine synthesis was carried out as described in section 2 using 1.65 pmol of 60 S subunits or particles derived from them and 1.65 pmol of standard 40 S subunits. The 100% value corresponds to 5.4 pmol of [^{14}C]phenylalanine incorporated. The EF-2-dependent GTPase activity was measured using 9.9 pmol of 60 S subunits or particles derived from them, 488 pmol of [γ-^{32}P]GTP (13 Bq/pmol) and 94 pmol of EF-2. Released [^{32}P]phosphate was extracted as phosphomolybdate with benzene/isobutanol (1:1) [4]. The 100% value corresponds to 96 pmol of GTP hydrolysed

the activities of the control 60 S subunit (not shown), stimulated the activities of the DMMA residual core particles to varying extents: 7 times the EF-2-dependent GTPase activity and 3 times the polyphenylalanine synthesizing activity. Almost identical values were obtained in five other experiments. The fact that stimulation of poly(U)-directed polyphenylalanine synthesis activity by P1–P2 was not as good as that of EF-2-dependent GTPase activity indicated that DMMA split proteins other than P1–P2 were needed for the interaction of the reconstituted large subunits with elongation factors, 40 S subunits, and aminoacyl tRNAs. This conclusion agrees with all the available data on the properties of the DMMA split proteins (see [4]). After treatment with insoluble alkaline phosphatase, P1–P2 lost their ability to reactivate the residual DMMA core particles in both the EF-2-dependent GTPase and the poly(U)-directed polyphenylalanine assays (table 1). This failure of the alkaline-phosphatase-treated extract to reactivate DMMA core particles could not be attributed to the inhibitory effect of residual alkaline phosphatase. Indeed the alkaline-phosphatase-treated extract neither hydrolyzed any *p*-nitrophenylphosphate at pH 9.5 nor inhibited control 60 S subunit activity (not shown). Interestingly, the treatment of the DMMA split protein fraction with alkaline phosphatase did not decrease the ability of this fraction to reactivate the DMMA core particles (table 1). This observation and more recent unpublished results strongly suggest that proteins P1–P2 are not free in the DMMA wash but belong to a complex, which protects them against inactivation by phosphatase.

The thermally induced hyperchromicity of rRNA within ribosomes is a convenient test of their conformational state [11]. If specific ribosomal proteins stabilize the ribosomal structure one can expect that the thermal denaturation patterns of core particles deprived of these proteins would differ from those of control ribosomal subunits and of reconstituted particles. As can be seen in fig.1 the DMMA residual particles showed a definite shift of the melting-out curve as compared with that of control subunits. In three experiments these particles started to melt at a lower temperature than the subunits: 46°C ± 0.5°C instead of 54°C ± 0.5°C. When the split proteins were added back to the particles, this temperature

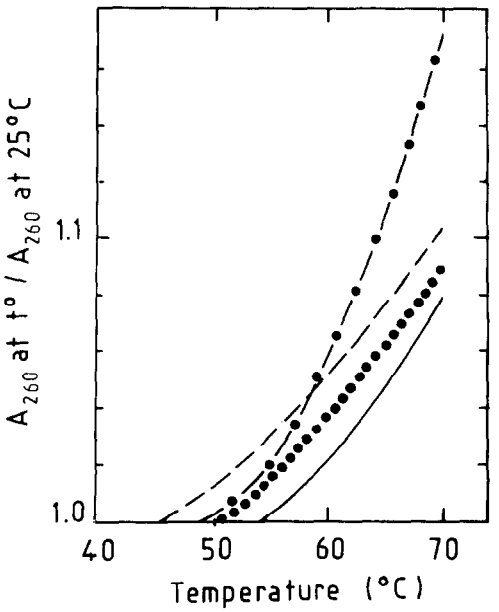

Fig.1. Influence of P1–P2 on the melting-out curve of 60 S ribosomal subunits. 1 A_{260} unit of control 60 S subunits (——), DMMA residual particles (– – –), subunits reconstituted with either the DMMA wash (●●●) or the ethanol extract (●——●), according to Method I described in section 2, were dialyzed overnight against 1 mM potassium phosphate, pH 7.4, 30 mM KCl, 1.5 mM $MgCl_2$ and heated at the rate of 1°C/min. The absorbance at 260 nm was recorded using a Gilford spectrophotometer equipped with a thermoprogrammer.

rose again to a value that lay between those of control subunits and DMMA particles (51°C ± 0.5°C). P1–P2 alone exerted a marked influence on the conformational stability of the subunits: the particles 'reconstituted' with P1–P2 started to melt at 49°C ± 0.5°C and showed a melting-out curve which was, to begin with, close to that of subunits reconstituted with the DMMA wash. Thereafter, the slope of the curve increased. Sedimentation analysis of these particles heated at temperatures higher than 57°C showed the presence of aggregates. Particles reassembled with alkaline-phosphatase-treated P1–P2 aggregated at about 40°C during heating (not shown).

Our observation that addition of P1–P2 alone to DMMA core particles restored most of the EF-2-dependent GTPase activity and half of the protein synthesis ability recovered when using the DMMA split-protein fraction recalls results ob-

tained with *E. coli* 50 S ribosomal subunits. Addition of L7–L12 alone to CsCl core particles derived from these subunits and lacking part of their structural proteins similarly restores the EF-G-dependent GTPase activity [12]. However, contrary to what was found with these bacterial core particles, the GTPase activity of our DMMA core particles was not stimulated by addition of 20% methanol (not shown).

The results concerning the activity of P1–P2, taken together with the fact that these proteins are also present in the supernatant and are exchangeable (see [13]), support the hypothesis that P1–P2 might contribute, as a control system, to the regulation of protein synthesis. The melting-out curves of subunits reconstituted with P1–P2 indicated that these proteins influenced the conformation of the DMMA core particles. We suggest, then, that P1–P2 could regulate the conformation of 60 S subunits during protein synthesis. As dephosphorylated P1–P2 gave rise to aggregative and inactive particles we could not assess the influence of phosphorylation on the conformation of the 60 S subunit. Most likely dephosphorylation lessens the solubility of P1–P2 which are particularly rich in hydrophobic amino acids [14]. It has been suggested recently that phosphorylation of the ribosomal acidic phosphoproteins regulates the binding of these to ribosomes [15].

Our findings are in agreement with the data on the functional significance of phosphorylated P1–P2 in EF-2-dependent GTPase activity and the polyphenylalanine synthesis ability of 80 S ribosomes, reported by MacConnell and Kaplan [3]. However, we would stress that our results are a significant extension of the former because we have used 60 S ribosomal subunits. This fact allowed us to get a better insight into the localization of P1–P2. We have shown that these proteins, like their equivalent L7–L12 [16], are remote from peptidyltransferases. Indeed their complete removal from the 60 S subunit by DMMA does not alter the puromycin reaction [4]. They are at or near the EF-2-binding domain with other relatively less active proteins: the extent of recovery of EF-2-dependent GTPase activity with the DMMA wash was only slightly higher than with P1–P2 alone. Other proteins in the same domain are probably A33 and L12 which, like P2, cross-link to EF-2 [17]. Like L7–L12, P1–P2 are close to 5 S RNA and should partly cover the contact surface between large and small subunits. Indeed P1–P2 have been found with another protein of the DMMA wash (L22), within a 5 S RNA-protein complex, able to associate to 40 S subunits [18]. Recently it has been suggested that P1–P2 form the mobile domain visible in rat liver 60 S ribosomal subunits which is analogous to the L7–L12 stalk within the *E. coli* 50 S ribosomal subunit [19].

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