Pivotal role of the P1 N-terminal domain in the assembly of the mammalian ribosomal stalk and in the proteosynthetic activity.

Philippe GONZALO (†), Jean-Pierre LAVERGNE and Jean-Paul REBOUD

Laboratoire de Biochimie Médicale, IBCP-UMR 5086 CNRS,
7, passage du Vercors, 69367 Lyon Cedex 07, France.

(†) To whom correspondence should be addressed: Philippe Gonzalo, IBCP-UMR 5086 CNRS, 7, passage du Vercors, 69367 Lyon Cedex 07, France. Tel.: (33) 0472722645; Fax: (33) 0472722605; E-mail: p.gonzalo@ibcp.fr.

Running title: Structure-function relationship in the ribosomal P-proteins

Keywords: reconstitution - refolding - protein interaction – ribosome - P-proteins - lateral stalk - GTPase center.
Summary
In the 60 S ribosomal subunit, the lateral stalk made of the P-proteins plays a major role in translation. It contains P0, an insoluble protein anchoring P1 and P2 to the ribosome. Here, rat recombinant P0 was overproduced in inclusion bodies and solubilized in complex with the other P-proteins. This method of solubilization appeared suitable to show protein complexes and revealed that P1, but not P2, interacted with P0. Further, the use of truncated mutants of P1 and P2 indicated that residues (1-63) in P1 connected P0 to residues (1-65) in P2. Additional experiments resulted in the conclusion that P1 and P2 bound one another, either connected with P0 or free as found in the cytoplasm. Accordingly, a model of association for the P-proteins in the stalk is proposed. Recombinant P0 in complex with phosphorylated P2 and either P1 or its (1-63) domain restored efficiently the proteosynthetic activity of 60 S subunits deprived of native P-proteins. Therefore, refolded P0 was functional and residues (1-63) only in P1 were essential. Besides, our results emphasize that the refolding principle used here is worth considering to solubilize other insoluble proteins.
Introduction

The ribosome is the central constituent of the protein synthesis machinery (1). During translation of messenger RNA into protein, it is helped by several soluble factors that operate in a sequential manner to improve both efficiency and fidelity of this process (2, 3). How this extraordinary coordination is performed is not yet exactly understood. Still, since most of the translation factors are GTPases, the driving of the factors by the ribosome is likely to be controlled by GTP hydrolysis (4). A small portion of the 28 S rRNA designated as the GTPase center is known to be involved in GTP hydrolysis activation (5). The GTPase center is connected directly to the stalk (6), an elongated and very flexible protuberance interacting with elongation factors (7-10). However, in spite of decades of research, organization and functions of the proteins constituting the stalk remain unclear. The number and the nature of the proteins constituting the stalk are different depending on the biological system although their general organization, made of five proteins, is likely to be similar. In prokaryotes, four identical proteins (L7/L12) are linked to the GTPase center by L10 connected itself with a sixth protein, L11 (11). In mammals, the equivalents of the four L7/L12 are two different proteins, P1 and P2, each being present in two copies. These proteins are bound to P0, the equivalent of L10, which is itself bound to L12, the eukaryotic equivalent of L11, and to the GTPase center (6, 12). In plants, an additional protein, P3, has been described (13). In yeast, there are two variants of both P1 (P1α and P1β) and P2 (P2α and P2β), the precise repartition and function of each variant remaining unsettled (14). This structural heterogeneity seems to correspond to functional differences and data obtained in one system cannot be extrapolated directly (10). Both L7/L12 and P1/P2 have in common their size (around 110-120 residues), their acidity, and the fact that amino- and carboxy-terminal domains are joined by an alanine-rich flexible region (15). The C-terminal protruding region (16, 17) is identical in P0, P1 and P2 and contains phosphorylation sites not found in prokaryotes. In the rat, P2 phosphorylation has been shown to stimulate the proteosynthetic activity of the ribosome (18) and the GTPase activity of eEF-2 (19). The N-terminal domains of P1 and P2, although of very different lengths in eukaryotes and prokaryotes, are involved in P0 and L10 binding, respectively (20, 21). In eukaryotes, an exchange between the ribosome-bound P1 and P2 (but not P0) and a cytoplasmic pool of these proteins has been shown (22-24), a situation not found with L7/L12 in prokaryotes. Functions and conditions of this exchange remain unexplained. P0, contrary to L10, contains the flexible alanine-rich region and the phosphorylatable C-terminal domain found in P1 and P2. Mobility complicates the study of the components of the stalk that represent two of the last three proteins not shown in the crystallographic structure of the 50 S ribosomal subunit of the archaea, Haloarcula marismortui (25, 26). Besides, structural studies of the isolated proteins are incomplete (27). This mobility is biologically relevant (28) and the conformation of the stalk was shown to be different depending on the step of the ribosomal cycle (8, 9, 29, 30). Hence, it is a major goal to understand how these dramatic conformational changes operate at a molecular level.
In previous works from our laboratory, rat recombinant P1 and P2 proteins were overproduced and studied when linked to the ribosome (18) and as isolated proteins (19). The lack of P0 prevented to go further in the study of their functions in a ribosomal context. Here, recombinant P0 was successfully overproduced and shown to be effective to reconstitute a functional stalk together with P1 and phosphorylated P2. Several approaches were used to determine the interactions between the stalk components and led us to propose a new model for its functional architecture.
Experimental Procedures

Materials -
The RNAgent Total RNA Isolation System kit, the oligo (dT)$_{15}$ used as a primer for the reverse transcription (RT), the Bam H I and Smal I restriction enzymes, the T4 DNA ligase and the E. coli cells (JM109 strain) were from Promega. The primers used for the DNA polymerase chain reactions (PCR) were from Isoprim. Pwo DNA polymerase, RNase H and β-octylglucoside were acquired from Boehringer-Mannheim. Superscript II, the reverse transcriptase RNase H', was from Gibco BRL Life Technologies. The pQE-30 plasmid and the Ni$^{2+}$-nitrilotriacetic acid-agarose gel (Ni-NTA) came from Qiagen. Preparation and properties of the monoclonal antibody (4C3) used to detect the P-proteins in the Western blot have been previously described (31).

Methods -
Construction of the P0-pQE-30 expression vector -
The cDNA of P0 was obtained by RT-PCR of total RNA prepared from 1 g of rat liver (Wistar strain) using the general guanidinium thiocyanate plus 2-mercaptoethanol method (32). Reverse transcription step was performed under Superscript II standard conditions. PCR (30 cycles) was performed using 0.2 µL of the reverse transcription product and 10 µM of the 5' (TAT GGA TCC ATG CCC AGG GAA GAC AGG GCG ACC) and 3' (TAT CCC CGG TTA GTC GAA GAG ACC GAA TCC CAT) primers. Annealing temperature was 59 °C. P0 cDNA (960 bp) was cloned (18) and its sequence was in full agreement with the Swiss-Prot P0 sequence of Rattus norvegicus (Entry name: RLA0_RAT; Primary accession number: P19945) corrected for three conflicts compared to the previously published sequence (33, 34).

Overproduction and purification of P0 -
P0, overproduced as described for P1 and P2 (18), was mostly insoluble and purified from inclusion bodies. The pellet of the bacterial lysate was submitted to a sequential extraction with 12 volumes of 1.5 M - 3 M and 6 M guanidine. P0, extracted in the 3 M and 6 M guanidine fractions, was eluted from a Ni-NTA-agarose gel chromatography with 190 mM imidazole in a buffer containing 4 M guanidine, 50 mM ammonium phosphate pH 7.5, 300 mM KCl, 0.1 mM EDTA, 10% (v/v) glycerol. After dialysis against this buffer, the preparation, divided into aliquots, was frozen at -80°C. The final yield from a one liter culture was 15 mg.

Cloning, overproduction and purification of the mutants of P1 and P2 -
Truncated mutants of P1 and P2 were used in this work. N1 and N2 comprise the amino acids 1 to 63 and 1 to 65 from P1 and P2, respectively. These proteins were overproduced after the cloning and sequencing of their cDNAs as described previously (18). cDNAs were obtained by PCR using P1-pQE-30 and P2-pQE-30 vectors as templates (18), the 5' primers previously described (18) and the following 3' primers (ATT AAG CTT TTA TAC ATT GCA GAT GAG GCT TCC for N1 and ATT AAG CTT TTA CAC ACT GGC CAG CTT GCC AAC for N2). N2 was overproduced in the supernatant only and purified as described for P1 and P2 (18). N1, found only in inclusion bodies, was
purified following the procedure used for P0 except that 8 M guanidine was necessary to solubilize and to purify it.

**Solubilization of P0 and N1**

Renaturation was carried out by removing the guanidine with an overnight dialysis at 4 °C. 10 µM of P0 (or 20 µM N1) and 20 µM of the ligand(s) (P1 alone or N1 plus either P2 or N2 in the case of P0; N2 or P2 in the case of N1) in 2 M guanidine, 40 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM 2-mercaptoethanol, 1 µM EDTA, and 20 % (v/v) glycerol were dialyzed against the same buffer but without guanidine. A similar procedure was used for N1 using P2 or N2 as ligands. The yields of solubilization of either P0 or N1 by increasing ligand concentrations were determined according to the following procedure. Mixtures containing a fixed concentration of either P0 or N1 were dialyzed with increasing ligand concentrations and then centrifuged at 17,000 × g for 30 min. In each test, the concentration of the proteins in the supernatant (solubilized P0 or N1, plus the ligands) was determined using the “Coomasie-blue plus protein assay” reagent kit from Pierce. Then, soluble P0 or N1 concentration was obtained by subtracting ligand concentration to the measured concentration. This calculation is well-founded since ligands behave as soluble proteins that were not found in the pellets in a significant manner after the renaturation process. These measures were checked by analyzing aliquots of both the supernatants and the pellets in a SDS-PAGE and by quantifying the bands corresponding to either P0 or N1 using a Personal Densitometer SI (Molecular Dynamics) equipped with the Image QuanNT software as previously described (19).

**Analysis of the complexes between the P-proteins by 2-D electrophoresis**

The supernatant of the dialyzed solutions of the P-proteins was loaded onto a non denaturing, 1.5 mm-thick, 6%-polyacrylamide electrophoresis gel (13 cm × 13 cm) cooled at 4 °C (35) that contained 50 mM Tris/HCl pH 8.5, 50 mM KCl, 5 mM β-octylglucoside, 5 mM MgCl₂, and 15 % (v/v) glycerol. A premigration was carried out during 1 hour at 80 V before loading the samples. The sample buffer contained 150 mM Tris/HCl pH 8.5, 300 mM KCl, 5 mM MgCl₂, 20 mM β-octylglucoside, 20 mM dithiothreitol (DTT), 20 % (v/v) glycerol and 0.02 % bromothymol blue. The overlay buffer poured onto samples was identical except that it contained only 10 % (v/v) glycerol and 50 mM KCl. Migration was performed at 100 V for 1 hour, 200 V for 2 hours and then, 250 V until the tracking dye reached the end of the gel. Electrophoresis buffer (150 mM Tris/HCl pH 8.5, 50 mM KCl, 5 mM MgCl₂ and 5 mM DTT) was changed every hour. The gel was Coomasie blue stained and the slab corresponding to the first electrophoresis was cut and included in the stacking layer of a 2 mm-thick 17%-SDS-polyacrylamide gel (36).

**Reconstitution of active DMMA-particles**

Extraction of P0 from 60 S subunits and reconstitution of the biological activity after addition of either the split or recombinant proteins was adapted from the method described by Nieto and coworkers (37) with the following modifications: 1) 60 S subunits and DMMA concentrations were 0.8 µM and 21.6
mM, respectively; 2) all steps were performed on ice; 3) split proteins were separated from the DMMA-particles by an ultracentrifugation through a 15% sucrose layer; 4) core particles and split proteins were dialyzed against a buffer containing Bis-Tris pH 6.0 in place of sodium cacodylate; 5) reconstitution was performed in the same buffer at pH 7.0; 6) proteins not bound to DMMA-particles were separated by a second ultracentrifugation through a 15% sucrose layer. The proteosynthetic activity of the subunits was measured by the polyphenylalanine synthesis test (38).
**Results**

*P0 is overproduced as an insoluble protein -*

P0 overproduced in an *E. coli* system fused with a N-terminal poly (His)-tag was found mostly in inclusion bodies contrary to what had been found previously with P1 and P2 overproduced under similar conditions (18). Co-production of P0 with the chaperonines GroES/GroEL or with thioredoxin did not improve the amount of soluble P0 in the bacterial supernatant. Hence, P0 was purified from inclusion bodies using 4 M guanidine. Several methods to obtain non-denatured soluble P0 from this solution were unsuccessful: dilution or dialysis of the guanidine under different conditions resulted in P0 precipitation as well as an attempt to refold it immobilized on the Ni-NTA affinity column used for its purification. This led us to try to renature P0 in the presence of its available potential ligands in the stalk, the soluble proteins P1 and P2.

*P1 but not P2 can solubilize P0 -*

P0 was mixed with recombinant proteins P1 and P2 in 2 M guanidine to keep it soluble. When guanidine was removed by dialysis (Figure 1), nearly all P0 remained soluble in the presence of P1 alone and (P1 + P2) but surprisingly, not in the presence of P2 alone (compare the insoluble proportion of P0 in lanes 2, 4 and 3, respectively, with that in lane 1 corresponding to P0 alone). The small amounts of P1 and / or P2 that were found in the pellets in lanes 2 to 4 were also found when P1 and /or P2 were submitted to the solubilization process in the absence of P0 (data not shown). This precipitation should originate from a very limited denaturation of P1 and P2 under the conditions of the solubilization process. This experiment suggested that P1 but not P2 could interact with P0 to make a soluble complex. Quantitative data were obtained and the effect of P1 concentration on P0 solubilization led to the determination of the stoichiometry of this complex (see Figure 4 below, black circles).

*Rationale for the design of shortened mutants of P1 and P2 -*

Since it had been suggested that N-terminal domains in P1 and P2 were involved in the binding to P0 in yeast (21), we have prepared truncated mutants of rat P1 and P2. N1 and N2, the N-terminal domains of P1 and P2, respectively, contained the 63 and 65 first residues of P1 and P2 preceded by a poly (His)-tag. N1 and N2 sequences are highlighted in grey in the Figure 2 in which hydrophobic regions are shown under the sequences. N2, predicted to be a very hydrophilic protein, was purified from the bacterial supernatant. In contrast, N1, mostly made of hydrophobic regions, was overproduced and purified only from inclusion bodies in 8 M guanidine.

*N1 interacts with both N2 and P0 -*

Dialyses of N1 in the presence of P2 or N2, which were potential ligands for N1, were carried out to determine whether it was possible to solubilize N1 in the absence of guanidine (Figure 3A). N1 a completely insoluble protein (lane 1) was solubilized by both N2 (lane 2) and P2 (lane 3). The effect of increasing concentrations of either N2 or P2 on N1 solubilization (20 µM) was studied (Figure 3B). For that, N1 solubilization was carried out in the presence of increasing concentrations of either N2 or
P2 and the concentrations of N1 remaining soluble after dialysis of the guanidine were determined using the procedure described in Experimental procedures and in the legend of Figure 3. Using either P2 or N2, we observed that soluble N1 concentration was directly proportional to that of P2 or N2 up to 20 µM. The shapes of these curves revealed equimolar complexes between N1 and P2 or N1 and N2 since the slopes were equal to one. Further, the level of the plateau indicated that the solubility of the complex N1-P2 (white circles) was 20 µM at least, this concentration corresponding to the solubilization of all the N1 available in the test. The complex N1-N2 (black diamonds) was slightly less soluble (about 18 µM) since a small fraction of N1 remained insoluble whatever the concentration of N2 tested. This result indicated that P1 and P2 could bind each other and that their N-terminal domains were involved in the process of heterodimerization. This suggested also that the common C-terminal domain might play a main part in the P-protein solubility, which might be due to its high hydrophilicity (see Figure 2). On the 2-D electrophoreses displayed in Figure 5, P1-P2 and N1-P2 complexes, respectively, were visualized as complexes C1 in panel A and C2 in panel B (see below for more details).

To determine the stoichiometry of the complexes between P0 and its ligands, an approach similar to that used for N1 in Figure 3B was applied to P0 (Figure 4). Using P2 as a potential ligand, no enhancement of P0 solubility was shown, regardless of the P2 concentration (black triangles). Using P1 as a ligand (black circles), concentration of soluble P0 increased linearly with P1 concentration with a slope of about 0.5 (µM of P0 / µM of P1) up to about 20 µM P1. This slope suggested that a complex containing two P1 molecules for one P0 was formed (P0 concentration in the test was 10 µM). Above 20 µM P1, a plateau corresponding to the solubilization of all the P0 available was observed. A similar result was obtained using (P1 + P2) in place of P1 alone. These results showed that two P1 molecules (or two heterodimers P1/P2) had to associate with P0 to solubilize it.

To test whether N1, the insoluble N-terminal domain of P1, interacted with P0, a trial of (N1 + P0) co-solubilization was performed (black squares in Figure 4). It resulted in no solubilization of P0 and even in a reduction of its solubility. This suggested that a complex between N1 and P0 was formed but was insoluble, which might originate from the fact that the hydrophobic regions constituting mainly P0 and N1 were not all buried in the complex (see Figure 2). Then, the ability of the complexes N1-P2 and N1-N2 to solubilize P0 was studied (Figure 4). Contrary to P2 or N2 alone (black triangles) that did not modify the solubility of P0, the addition of increasing concentrations of either (N1 + P2) (white circles) or (N1 + N2) (black diamonds) resulted in a substantial increase in P0 solubility. These observations indicated that P2 did not bind to P0 directly but through an interaction between its N-terminal domain and that of P1. Still, the shapes of the curves suggested that these complexes were less efficient than full-length P1 (or (P1 + P2)) to achieve P0 solubilization. Indeed, the maximal effect was obtained with a higher molecular ratio (around three versus around two) suggesting that P0 affinity for N1-P2 or N1-N2 was lower than that for P1 or P1-P2. Further, the maximal solubility of these complexes was lower (around 6.5 µM) than that given by P1 (or (P1 + P2)) (at least 10 µM: the
highest solubility was not obtained). These data would indicate that the hinge regions of P0 and P1 and perhaps also the C-terminal domains could be involved in the stabilization of the complex P0-P1.

The existence of the complex resulting from the association of P0 with P2 through the N-terminal domain of P1 was directly shown on a 2D-electrophoresis of a mixture of P0, N1 and P2 (Complex C3 in Figure 5B). A significant part of P0 precipitated in the well (L) probably owing to the low salt-concentration in the 1st-D electrophoresis buffer and to a stabilization of the aggregates by oxidation of the P0 cysteine. P2 migrated in excess compared to P0 and N1 since about 40% of P0 and N1 had precipitated during the dialysis (Figure 4, white circles). The free form of P2 was found to migrate as the broad band labeled (F), which might originate from an association / dissociation equilibrium of P2 dimers under these conditions (See below and (17)).

Incorporation of P1 and P2 into the ribosomal stalk requires the prior formation of P1-P2 dimers -

The preceding results led us to conclude that P1 and P2 associated as heterodimer to P0. However, the question could be asked whether P1 and P2 could form homodimers and whether these homodimers might participate in the stalk formation?

To answer these questions, N1, the insoluble N-terminal domain of P1, was mixed with either full-length P1 (lane 1) or P2 (lane 2) (Figure 6A): the amount of solubilized N1 was approximately equal in both lanes. From this experiment, one could conclude that P1 associated significantly to N1, and hence, that two P1 (or more) could bind by their N-terminal domains. The ability of P2 to dimerize was deduced from its behavior when passed through a gel filtration column: it was eluted as a single peak, the elution volume of which corresponding to the mass of a P2 dimer (data not shown).

However, by the 2D-electrophoresis of the equimolar mixture of P1-P2 (Figure 5A), it was shown that all the proteins were involved in the P1-P2 complex. Indeed, nearly no P2 was found in the broad band F corresponding to unbound P2 (compare with panel B). This indicated that in the absence of P0, purified P1 and P2 bound one another to form heterodimers and that there was a disruption of the homodimers.

To elucidate whether P1 and P2 could bind P0 as homodimers, we mixed the two insoluble proteins, P0 and N1 (molar ratio 1:2), in the presence of either P1 or P2 (molar ratio to P0 1:2) (Figure 6B). We observed that in both cases, a part of P0 and N1 was solubilized but in different proportions. In the presence of P1 (lane 1), a large amount of P0 was solubilized whereas the majority of N1 had precipitated (compare the ratio P1 to N1 in panel A with that found in panel B). In the presence of P2, the situation was the opposite (compare lane 2 with lane 1 in panel B): the amount of solubilized P0 was lower and that of N1 was higher. From these results, one could deduce that P1 bound poorly to N1 in the presence of P0. Consequently, this experiment suggested that the N-terminal domain of P1 had a common binding site for both P0 and P1 and therefore that it could bind simultaneously only P0 and the N-terminal domain of P2 but not a second P1. Therefore, it was likely that the P1 homodimers observed in purified solution had to dissociate to allow the binding to P0 in the stalk. The binding of the N-terminal domain of P2 to that of P1 explained the situation illustrated in lane 2: P2 had to
associate first with N1 and the N1-P2 complex has been already shown to be less effective to solubilize P0 than P1 alone in the experiments reported in Figure 4 (white circles).

**60 S subunits reconstituted using the three recombinant proteins are active -**

Since both P0 and N1 were solubilized recombinant proteins, it was required to test whether they were functional. This was accomplished by measuring the proteosynthetic activity of 60 S subunits in which native P0, P1 and P2 have been removed and replaced by the recombinant proteins. Extraction of the native P-proteins was performed by adapting a long-established method utilizing dimethylmalleic anhydride (DMMA) (37). Under classical conditions (molar ratio DMMA to 60 S equal to 15,000), P1 and P2 were entirely extracted contrary to P0 and a molar ratio above this value resulted in an inability to restore the DMMA-particle proteosynthetic activity (38). Here, we used a 27,000 molar ratio but under milder experimental conditions (See Experimental Procedures for details). As shown in the immunoblot revealing P0, P1, and P2 (Figure 7), there was no P0 left in DMMA-particles obtained under these new conditions (lane 2). The residual amount of P1 in this lane in the absence of P0 might be due to the high salt concentration of the extracting buffer that reinforced unspecific hydrophobic interactions and could make P1 stick to the DMMA-particles. A comparison of the ratio P0 to (P1 + P2) in native subunits (lane 1) with that found in the split proteins before dialysis (lane 3) indicated that some P0 had precipitated during the extraction process. A comparison of these ratio before (lane 3) and after dialysis (lane 4) showed that soluble remaining P0 ended precipitating during the dialysis needed to regenerate the amino groups of the proteins removed by the DMMA extraction. This precipitation was likely to be due to the disruption of the complex P0-P1 by the DMMA treatment and explained why the use of a molar ratio of DMMA to 60 S above 15,000 (classical conditions) resulted in the inability to reactivate the DMMA-particles with the split proteins. Indeed, results in table I showed that the DMMA-particles lacking P0 were poorly reactivated when reconstituted with either the split proteins (27%) or a mixture of recombinant P1 and phosphorylated P2 (P2p) (26%). Addition of P0 to the last mixture restored most of the activity (83%). Interestingly, a mixture containing P0, P2p and N1 instead of P1 was shown to reactivate the DMMA-particle (80%) as well as the mixture containing full-length P1. From these results, one could conclude that both recombinant P0 and N1 were functional after solubilization and in addition that the intermediary and C-terminal domains of P1 were dispensable for the proteosynthetic activity of the ribosome.
Discussion

The experiments presented here were designed to elucidate how the acidic ribosomal proteins P0, P1 and P2 associate into the stalk of the mammalian ribosome and the role of each component. To fulfill this aim, we have overproduced recombinant P0 in addition to P1 and P2, which had already been obtained and studied as isolated proteins (18, 19). Getting P0 has made possible to study the association of the P-proteins as they are in the stalk. Truncated mutants of the latter proteins were designed and prepared to locate the binding domains and to study their functions.

Biological activity of recombinant P0 -

The fact that recombinant P0 was overproduced in inclusion bodies, contrary to P1 and P2, the other ribosomal stalk components, raised the question whether it was functional after being refolded. Therefore, a method to assess the biological activity of recombinant refolded P0 was developed and showed that P0, in addition to bind P1, was able to reconstitute efficiently the proteosynthetic activity of ribosomes deprived from native P0 (Figure 7 and Table I). It is noteworthy that native P0 has been previously reported to be insoluble (39) in agreement with our observation (Figure 7) and the fact that no P0 is found in the cytoplasmic pool of mammalian cells (24). Therefore, the observed insolubility of P0 is likely an intrinsic property of the protein and not a consequence of its misfolding.

P0 interacts with P1 but not with P2 -

Our results indicate that only P1 forms a stable complex with P0. The stoichiometry of the complex is two P1 molecules for one P0, which strongly suggests that P0 solubilization by P1 does not involve an unspecific interaction (Figure 4). No complex between P0 and P2 is shown in our experiments, neither in the solubilization experiments (Figures 1 and 4), nor in the 2-D electrophoresis (Figure 5B). Hence, it can be concluded that P1 and P2 play a different part in the formation of the stalk. Such conclusion is in agreement with previous results obtained with rat liver P1 and P2 (40) and with recent results showing that P2β is unable to bind to P0 contrary to P1α in Saccharomyces cerevisiae (41).

The N-terminal domain of P1 (N1) interacts with both P0 and the N-terminal domain of P2 (N2) -

Experiments made with N1 indicate that this domain binds both P0 and P2 (Figures 3-6). N1 is a small protein (63 residues), which suggests that the N-terminal part of P1 has evolved to promote specific interactions with both P0 and P2. This might explain why P1 proteins from different species do not replace each other in spite of important sequence homology (42). The fact that P1-P2 association involves mainly their N-terminal domains (Figure 3B) is intriguing since N1 is mainly hydrophobic whereas N2 is mainly hydrophilic (see Fig. 2). Moreover, P1 and P2 interaction differs from that found in L7/L12 in which both the intermediate and C-terminal domains participate in the dimerization by burying hydrophobic groups at the dimer interface (27). Because sequence identities are too low (15%) between L7/L12 and P1 or P2, no reliable model of the N1/N2 interaction using the available L7/L12 coordinate file (PDB 1DD4) can be built (27). Here, we do not exclude that the hinge and the C-terminal domains are also involved in the dimerization process but our data indicate that they have not a prominent function (Table I). In contrast, concerning the binding of P1 to P0, P1
intermediary (and perhaps also C-terminal) domains might be involved in the binding more significantly since affinity and solubility of N1-containing complexes might be lower than those of the complexes involving full-length P1 (Figure 4). However, DMMA-particles reconstituted with either N1 or P1 and both P0 and phosphorylated P2 (P2p) have similar proteosynthetic activities. Therefore, the deleted hinge and C-terminal charged domains of P1 should not play a prominent function in protein synthesis and in the interaction of the ribosome with eEF-1α and eEF-2, the two elongation factors required in the in vitro poly (U) directed-poly (Phe) synthesis test.

*New models of association of the P-proteins in the stalk -*

P1 has been shown to interact by its N-terminal (1-63) domain both with P0 and the N-terminal (1-65) domain of P2. In addition, no direct interaction was shown between P0 and P2, albeit it should have been revealed by the different methods used. Thus, between the three possible models of the stalk that can be drawn (Figure 8), model A (38) is inconsistent with our experimental data since its is based on the assumption that both P1 and P2 bind to P0 and are present as homodimers. Here we present several data indicating that there is a disruption of the homodimers of both P1 and P2 (observed only in purified solutions in our experiments) to constitute P1/P2 heterodimers (Figures 3, 5 and 6). That heterodimers of human P1/P2 can be formed more easily than homodimers of P1 and P2 has been recently reported (44). P1 binds preferentially P0 rather than N1 and is seemingly unable to bind them simultaneously (Figure 6B). Therefore, model C only would be in agreement with our results. Model C would be also in agreement with results obtained for the prokaryotic model in which L10 is shown to have distinct binding sites for each dimer of L7/L12 (43). The association of P1 and P2 into heterodimers either free (as they are in the cytoplasm) or bound to P0 (as found in the stalk) suggests that P1 and P2 bind directly to the ribosome as heterodimers and not sequentially. However, in previous work, the presence of P1 and P2 homodimers in the stalk has been suggested (40). Large modifications in the conformation of the stalk in response to factor binding or changes in the A-site or the P-site of the ribosome have been observed (8, 9, 29, 30). Then, model B that simulates a conformation of the stalk in agreement with the presence of both homodimers and heterodimers of P1 and P2 and, model C, in agreement with our own results, might represent two sequentially existing structures of the stalk.

*Production of functional recombinant proteins from inclusion bodies -*

In addition to give precise and relevant details on the organization of the lateral stalk of the mammalian ribosome, this study emphasizes the interest of using protein ligands to promote functional refolding of recombinant insoluble proteins. Indeed, a similar procedure applied to refold both N1, the insoluble N-terminal domain of P1, and P0 gave functional proteins (Table I). That disordered fragments of the same protein reconstitute the native structure upon association has already been shown (45) and would arise from a molecular recognition between disordered polypeptide chains in a process coupling association with folding (46, 47). Here, we have adapted this general principle to the
folding of different interacting proteins. This approach might be of general interest to promote the functional refolding of recombinant insoluble proteins.
Figure 1: Differential effects of P1 and P2 on P0 solubilization.
Solubilization of 28 µg (0.8 nmol) of P0 (35.2 kDa) was performed by an overnight dialysis of the guanidine in the absence of any other protein (lane 1) or in the presence of 1.6 nmol (21 µg) of either P1 alone (12.9 kDa) (lane 2) or P2 alone (13.1 kDa) (lane 3), or in the presence of 1.6 nmol of (P1 + P2) (lane 4). After centrifugation, the proteins contained in the pellets were analyzed in a 15%-SDS-PAGE (36). P1 and P2 are known to migrate at a higher molecular mass than predicted from their sequence.

Figure 2: Location of the hydrophobic regions in P0, P1 and P2.
Hydrophobicity of the P-proteins was studied following the method of Kyte and Doolittle (48) using MPSA, a software available at http://www.ibcp.fr (49, 50). Hydrophobic regions are displayed with a + under the protein sequence. The common C-terminal sequence is written in bold black characters and the alanine/proline rich region (the hinge) is shown in grey bold letters. Sequences of the (1-63) and (1-65) N-terminal domains of P1 and P2, respectively, are highlighted in grey.

Figure 3: Effects of P2 or N2, the (1-65) domain of P2 on the solubilization of N1, the (1-63) domain of P1.
A: Solubilization of 16 µg (2 nmol) of N1 (8.0 kDa) in 100 µL was performed by an overnight dialysis of the guanidine in the absence of any protein (lane 1) and in the presence of an equimolar amount of N2 (8.0 kDa) (lane 2) or P2 (lane 3). After centrifugation, insoluble proteins were loaded in a 18%-SDS-PAGE (36). N2 (as P2) was a fully soluble protein under these conditions and does not contribute to the band shown in lane 2. N1 alone (lane 1) is a fully insoluble protein under these conditions.
B: Solubilization in 100 µL of 2 nmol of N1 (16 µg) was performed by an overnight dialysis of the guanidine using increasing concentrations of N2 (◆) or P2 (). After centrifugation, concentration of soluble N1 was measured by subtracting N2 or P2 concentration to that of the total soluble protein concentration since both N2 and P2 were fully soluble. In the case of P2, the accuracy of this method was verified by quantifying the bands corresponding to N1 in the pellet and in the supernatant after separation in a SDS-PAGE as described in Experimental procedures and (19). Using N2, this method could not be applied to quantify N1 in the supernatants since N1 and N2 had the same molecular weight.

Figure 4: Effects of increasing concentrations of potential ligands on P0 solubilization.
Solubilization of 1 nmol of P0 (35 µg) in 100 µL was performed by removing the guanidine with an overnight dialysis in the presence of increasing concentrations of various ligands.
After centrifugation, concentration of soluble P0 was measured by subtracting the ligand concentration to that of the total soluble protein concentration. Further, results given by this calculation were
assessed by quantifying the bands corresponding to P0 in the pellets and in the supernatants after separation in a SDS-PAGE as described in Experimental procedures and (19). When two proteins were added together, they were in equal molar concentration. The proteins tested for P0 solubilization were P1 (●); P2 (▲); N1 (■); (N1 + P2) (○); (N1 + N2) (◆). (P1 + P2) gave a curve superposable with that given by P1 alone and that of N2 was identical to that of P2.

Figure 5: Two-dimensional gel electrophoreses of P protein complexes.
A: An equimolar mixture of P1 and P2 was loaded in a non-denaturing 6%-PAGE at pH 8.5 and its migration is represented by the horizontal slab stained with Coomasie-blue. This slab was cut and included in the staking layer of an 18% SDS-PAGE (36). The 2nd-D electrophoresis reveals that P1 and P2 migrates mainly as a complex (C1) and that nearly no P2 is found in the broad band F containing free P2 (compare with panel B).
B: After dialysis and centrifugation, the soluble fraction of a P0, N1, and P2 mixture (molar ratio 1:2:2) was separated in bands L, C3 and C2 using a non-denaturing 6%-PAGE at pH 8.5 stained with Coomasie blue. L, at the basement of the well contained aggregated proteins unable to enter into the gel. The 1st D slab was cut and included into the staking layer of a 18%-SDS-PAGE (36). After migration and staining with silver nitrate, the 2nd-D-gel showed that L contained only P0, that C3 was a complex of P0 with N1 and P2 and, that C2 was a complex of N1 with P2. P0 and N1 that are insoluble did not migrate in the absence of P2 under these non-denaturing conditions. The broad band F represents the migration of free (unbound) P2 (see Results).

Figure 6: Compared effects of P1 and P2 on the solubilization of N1, the N-terminal domain of P1, either alone (A) or in the presence of P0 (B).
A: Solubilization of 19 µg (2.4 nmol) of N1 was performed by an overnight dialysis of the guanidine in the presence of an equivalent molar amount (31 µg) of P1 (lane 1) or P2 (lane 2) in 100 µL. After centrifugation, soluble proteins were loaded in a 18%-SDS-PAGE (36). N1 alone is a fully insoluble protein.
B: Solubilization of 0.6 nmol of P0 (21 µg) and 1.2 nmol of N1 (9.6 µg) was performed under the same conditions in the presence of 1.2 nmol (15.5 µg) of either P1 (lane 1) or P2 (lane 2) in 50 µL. After centrifugation, soluble proteins were loaded in a 18%-SDS-PAGE (36).

Figure 7: Immunodetection of the P-proteins in the DMMA-particles and in the split proteins.
DMMA-particles were prepared as described in Experimental Procedures. After proteins separation by a 15%-SDS-PAGE (36) and transfer on a nitrocellulose membrane, the content of P-proteins was determined using a monoclonal antibody probing the common C-terminal epitope (31). Lane 1, native 60 S; lane 2, DMMA-particles; lane 3, split proteins before the dialysis required for the regeneration of
amino groups; lane 4, split proteins after this dialysis. The amounts of DMMA-particles and split proteins corresponded to the amount of 60 S which they derived from.

Figure 8: Models of interaction of the ribosomal proteins of the stalk.

P0, P1 and P2 are represented, in spotted white, white, and grey, respectively. The C-terminal domain and the hinge are structural features common to the three proteins. Model A, built according to a previously proposed model (40), is not in agreement with our results since we have seen no direct interaction between P2 and P0. Model B differs from model C by the presence of additional interactions between P1 molecules. Model C only would be in agreement with our experimental data suggesting strongly that a P1 molecule cannot bind simultaneously another P1 molecule and P0 (see Figure 6).
References -
1. Pestka, S. (1968) J. Biol. Chem. 243, 2810-2820.
2. Preiss, T., and Hentze, M. W. (1999) Curr. Opin. Genet. Dev. 9, 515-521.
3. Ibba, M., and Soll, D. (1999) Science 286, 1893-1897.
4. Czworkowski, J., and Moore, P. B. (1996) Prog. Nucleic Acid. Res. Mol. Biol. 54, 293-332.
5. Uchiumi, T., Traut, R. R., Elkorn, K., and Kominami, R. (1991) J. Biol. Chem. 266, 2054-2062.
6. Uchiumi, T., and Kominami, R. (1992) J. Biol. Chem. 267, 19179-19185.
7. MacConnell, W. P., and Kaplan, N. O. (1980) Biochem. Biophys. Res. Commun. 92, 46-52.
8. Stark, H., Rodnina, M. V., Rinke-Appel, J., Brimacombe, R., Winterrmeyer, W., and van Heel, M. (1997) Nature 389, 403-406.
9. Agrawal, R. K., Penczek, P., Grassucci, R. A., and Frank, J. (1998) Proc. Natl Acad. Sci. U. S. A. 95, 6134-6138.
10. Gomez-Lorenzo, M. G., Sparn, C. M., Agrawal, R. K., Grassucci, R. A., Penczek, P., Chakraburtty, K., Ballesta, J. P., Lavandera, J. L., Garcia-Bustos, J. F., and Frank, J. (2000) Embo J. 19, 2710-2718.
11. Highland, J. H., and Howard, G. A. (1975) J. Biol. Chem. 250, 813-814.
12. Saenz-Robles, M. T., Vilella, M. D., Pucciarelli, G., Polo, F., Remacha, M., Ortiz, B. L., Vidalies, F. J., and Ballesta, J. P. (1988) Eur. J. Biochem. 177, 531-537.
13. Szick, K., Springer, M., and Bailey-Serres, J. (1998) Proc. Natl Acad. Sci. U. S. A. 95, 2378-2383.
14. Ballesta, J. P. G., Guarinos, E., Zurdo, J., Parada, P., Nusspaumer, G., Lalioti, V. S., Perez-Fernandez, J., and Remacha, M. (2000) in The ribosome: structure, function, antibiotics, and cellular interactions (R. A. Garret, S. R. D., A. Liljas, A. T. Matheson, P.B. Moore, and H.F. Noller, ed), p. 115-125, ASM Press, Washington, D.C.
15. Bushuev, V. N., Gudkov, A. T., Liljas, A., and Sepetov, N. F. (1989) J. Biol. Chem. 264, 4498-4505.
16. Hamman, B. D., Oleinikov, A. V., Jokhadze, G. G., Bochkariov, D. E., Traut, R. R., and Jameson, D. M. (1996) J. Biol. Chem. 271, 7568-7573.
17. Hamman, B. D., Oleinikov, A. V., Jokhadze, G. G., Traut, R. R., and Jameson, D. M. (1996) Biochemistry 35, 16680-16686.
18. Vard, C., Guillot, D., Bargis, P., Lavergne, J. P., and Reoubd, J. P. (1997) J. Biol. Chem. 272, 20259-20262.
19. Bargis-Surgey, P., Lavergne, J. P., Gonzalo, P., Vard, C., Filhol-Cochet, O., and Reoubd, J. P. (1999) Eur. J. Biochem. 262, 606-611.
20. Schop, E. N., and Maassen, J. A. (1982) Eur. J. Biochem. 128, 371-375.
21. Jose, M. P., Santana-Roman, H., Remacha, M., Ballesta, J. P., and Zinker, S. (1995) Biochemistry 34, 7941-7948.
22. Zinker, S., and Warner, J. R. (1976) J. Biol. Chem. 251, 1799-1807.
23. Mitsui, K., Nakagawa, T., and Tsurugi, K. (1988) J. Biochem. (Tokyo) 104, 908-911.
24. Tsurugi, K., and Ogata, K. (1985) J. Biochem. (Tokyo) 98, 1427-1431.
25. Ban, N., Nissen, P., Hansen, J., Capel, M., Moore, P. B., and Steitz, T. A. (1999) Nature 400, 841-847.
26. Ban, N., Nissen, P., Hansen, J., Moore, P. B., and Steitz, T. A. (2000) Science 289, 905-920.
27. Wahl, M. C., Bourenkov, G. P., Bartunik, H. D., and Huber, R. (2000) Embo J. 19, 174-186.
28. Dey, D., Bochkariov, D. E., Jokhadze, G. G., and Traut, R. R. (1998) J. Biol. Chem. 273, 1670-1676.
29. Agrawal, R. K., Penczek, P., Grassucci, R. A., Li, Y., Leith, A., Nierhaus, K. H., and Frank, J. (1996) Science 271, 1000-1002.
30. Agrawal, R. K., Heagle, A. B., Penczek, P., Grassucci, R. A., and Frank, J. (1999) Nat. Struct. Biol. 6, 643-647.
31. Fabien, N., Moreira, A., Lavergne, J. P., Desbos, A., Surgey, P., Alves de Olivera, C., Gonzalo, P., Venot, A., Bienvenu, J., Perrier, H., Reoubd, J. P., and Monier, J. C. (1999) J. Autoimmun. 13, 103-110.
32. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294-5299.
33. Wool, I. G., Chan, Y. L., Gluck, A., and Suzuki, K. (1991) Biochimie 73, 861-870.
34. Bairoch, A., and Apweiler, R. (2000) Nucleic Acids Res. 28, 45-48.
35. Madjar, J. J., Arpin, M., and Reboud, J. P. (1977) Anal. Biochem. 83, 304-310.
36. Laemmli, U. K. (1970) Nature 227, 680-685.
37. Nieto, M. A., Hernandez, F., and Palacian, E. (1989) Mol. Cell. Biochem. 86, 55-63.
38. Conquet, F., Lavergne, J. P., Paleologue, A., Reboud, J. P., and Reboud, A. M. (1987) Eur. J. Biochem. 163, 15-20.
39. Uchiumi, T., and Kominami, R. (1997) J. Biol. Chem. 272, 3302-3308.
40. Uchiumi, T., Wahba, A. J., and Traut, R. R. (1987) Proc. Natl Acad. Sci. U. S. A. 84, 5580-5584.
41. Zurdo, J., Parada, P., van den Berg, A., Nusspaumer, G., Jimenez-Diaz, A., Remacha, M., and Ballesta, J. P. (2000) Biochemistry 39, 8929-8934.
42. Bermejo, B., Prieto, J., Remacha, M., Coloma, A., and Ballesta, J. P. (1995) Biochim. Biophys. Acta 1263, 45-52.
43. Griaunova, O., and Traut, R. R. (2000) Biochemistry 39, 4075-4081.
44. Tchorzewski, M., Boldyreff, B., Issinger, O., and Grankowski, N. (2000) Int. J. Biochem. Cell. Biol. 32, 737-746.
45. Tasayco, M. L., and Chao, K. (1995) Proteins 22, 41-44.
46. Yang, X. M., Georgescu, R. E., Li, J. H., Yu, W. F., Haierhan, and Tasayco, M. L. (1999) Pac. Symp. Biocomput., 590-600.
47. Chaffotte, A. F., Li, J. H., Georgescu, R. E., Goldberg, M. E., and Tasayco, M. L. (1997) Biochemistry 36, 16040-16048.
48. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105-132.
49. Blanchet, C., Combet, C., Geourjon, C., and Deleage, G. (2000) Bioinformatics 16, 286-287.
50. Combet, C., Blanchet, C., Geourjon, C., and Deleage, G. (2000) Trends Biochem. Sci. 25, 147-150.
Footnotes -
Financial support came from the CNRS-UMR 5086 and both the Université Claude Bernard-Lyon I and the Hospices Civils de Lyon are also acknowledged.

Abbreviations used in this manuscript are: N1 and N2, the (1-63) and (1-65) domains of P1 and P2 proteins, respectively; Bis-Tris, [bis(2-hydroxyethyl)imino-tris(hydroxymethyl) methane]; DMMA, dimethylmaleic anhydride; P2p, phosphorylated P2; Ni-NTA, Ni²⁺-nitrilotriacetic acid-agarose gel., PCR, DNA polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

Acknowledgements -
We are grateful to Dr. D. Mandelman and Dr. J.-M. Jault for critical reading and commenting of the manuscript.
Figure 2:

|     | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 |
|-----|----|----|----|----|----|----|----|----|----|-----|
| PO  | MPREDRAIWKNSYFLKIIQGLDDYPRCFIVGDNWVGSKRQMQR | | | | | | | | | |
| Hy  | ++++++ ++++++ ++++++ ++++++ ++++++ ++++++ ++++++ ++++++ ++++++ ++++++ |
| P1  | | | | | | | | | | |
| P2  | | | | | | | | | |

|     | 110 | 120 | 130 | 140 | 150 | 160 | 170 | 180 | 190 | 200 |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| PO  | MLLANKVPAAARAGATAPCEFTVPAQNTGLGPEKTSFQALGITTKISRTEITLSDYQLITKGDXVGAESTALTINMNINSFSGTLTQQVFEDNGSTYS | | | | | | | | | |
| Hy  | +++  ++++++ ++++ ++++++ ++++++ ++++++ ++++++ ++++++ ++++++ ++++++ |
| P1  | | | | | | | | | | |
| P2  | | | | | | | | | |

|     | 210 | 220 | 230 | 240 | 250 | 260 | 270 | 280 | 290 | 300 |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| PO  | FVELDITEQALHTRFEQGVKAVSACOLLQIGYETVAVPHSIIINGYKRLALSVEQDYTFPLAEXKVMLADPSAFAAAPPVARATAAAPARAAPAKVEA | | | | | | | | | |
| Hy  | ++++ ++++++ ++++++ ++++++ ++++++ ++++++ ++++++ ++++++ ++++++ ++++++ |
| P1  | ---MASVSELAICYLHLDDBVTVNTEKINAIKACVNYHVPFPGLFAKALANVHICGTLQVAGCGPABAAGAPSGPASAAAPAEKKVEAK |
| P2  | ---MRYVASYLAACGNHPSPASKDITKTLDSUGTFAADDRNKNYTSFNLGKNTEDVTAQGCGLASVPPAGAVSAAPAPSAAPAPAEEKKDEK |
| Hy  | ++++ ++++++ ++++++ ++++++ ++++++ ++++++ ++++++ |

|     | 310 |
|-----|-----|
| PO  | KEESESEDMDGFGLFD 317 |
| Hy  | ++++ |
| P1  | KEESESEDMDGFGLFD 114 |
| Hy  | +++ |
| P2  | KEESEESDDMDGFGLFD 115 |
| Hy  | ++ |
Figure 3:

Panel A

| MW (kD) | 1 | 2 | 3 |
|---------|---|---|---|
|         | 14.4 | 8.2 | 6.2 |
|         | 2.5 |    |    |

Panel B

Soluble N1 concentration (µM)

N2 or P2 concentration (µM)
Figure 4:
Figure 5 (Panel A):
Figure 6:

Panel A

Panel B
Figure 7:

MW (kD) 1  2  3  4

33  20  14

P0

P1

P2
Figure 8:

Model A  Model B  Model C

Common C-terminal domain
Hinge
N1 and N2
P1/P2 binding domain of P0
rRNA binding domain of P0
TABLE I:

Proteosynthetic activity of 60S subunits reconstituted from recombinant P proteins and DMMA-particles:

| Reconstitution                        | Poly(Phe) synthesis (pmol/pmol) |
|---------------------------------------|----------------------------------|
| Control 60 S                          | 12.3 ± 1.8                       |
| DMMA-particles + Split Proteins       | 3.3 ± 0.7                        |
| DMMA-particles + (P1-P2p)$_2$         | 3.2 ± 1.0                        |
| DMMA-particles + P0 (P1-P2p)$_2$      | 10.2 ± 0.6                       |
| DMMA-particles + P0 (N1-P2p)$_2$      | 9.8 ± 0.4                        |

Proteosynthetic activities were measured under conditions in which subunits were limiting (38) and expressed in pmol of polymerized phenylalanine per pmol of either reconstituted particles or control 60 S after subtraction of the residual DMMA-particles activities (4.8 ± 0.8 pmol/pmol). Control 60 S subunits were treated the same way as DMMA-particles but without DMMA. Split proteins corresponded to the proteins displayed in lane 4 of Figure 7. Values were calculated from 6 different experiments. L-[14C]-phenylalanine specific activity was 20 Bq/pmol. In these experiments, P2 previously phosphorylated was used since such phosphorylation increased the activity of reconstituted particles (18).
Pivotal role of the P1 N-terminal domain in the assembly of the mammalian ribosomal stalk and in the proteosynthetic activity
Philippe Gonzalo, Jean-Pierre Lavergne and Jean-Paul Reboud

J. Biol. Chem. published online March 26, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101398200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts