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Topography and stoichiometry of acidic proteins in large ribosomal subunits from *Artemia salina* as determined by crosslinking

(protein synthesis)

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ABSTRACT **The 60S subunits isolated from *Artemia salina* ribosomes were treated with the crosslinking reagent 2-iminothiolane under mild conditions. Proteins were extracted and fractions containing crosslinked acidic proteins were obtained by stepwise elution from CM-cellulose. Each fraction was analyzed by "diagonal" (two-dimensional nonreducing–reducing) NaDodSO$_4$/polyacrylamide gel electrophoresis. Crosslinked proteins below the diagonal were radioiodinated and identified by two-dimensional acidic urea–NaDodSO$_4$ gel electrophoresis. Each of the acidic proteins P1 and P2 was crosslinked individually to the same third protein, P0. The fractions containing acidic proteins were also analyzed by two-dimensional nonequilibrium isoelectric focusing–NaDodSO$_4$/polyacrylamide gel electrophoresis. Two crosslinked complexes were observed that coincide in isoelectric positions with monomeric P1 and P2, respectively. Both P1 and P2 appear to form crosslinked homodimers. These results suggest the presence in the 60S subunit of (P1)$_2$ and (P2)$_2$ dimers, each of which is anchored to P0. Protein P0 appears to play the same role as L10 in *Escherichia coli* ribosomes and may form a pentameric complex with the two dimers in the 60S subunits.**

The large ribosomal subunits of *Escherichia coli* contain multiple copies of a protein, L7/L12, that is present as two dimers, each of which is anchored to the ribosome through interaction with a common protein, L10 (1–4). The four copies of L7/L12 are easily and selectively removed from the ribosome and can be reconstituted (5). The proteins are involved in the GTP-dependent reactions of protein synthesis—in particular, translocation (6–8). A model has been proposed recently, showing different orientation for the two L7/L12 dimers on the ribosome surface (9).

The large subunits of eukaryotic ribosomes also contain multicopy acidic proteins (10, 11) that are structurally and functionally related to L7/L12 (12–15). These proteins exist in phosphorylated states and have been designated P proteins (16–18). Two different P proteins having slightly different amino acid compositions, electrophoretic mobilities, and molecular weights have been designated P1 and P2 (16). Anti-ribosome antibodies from certain patients with systemic lupus erythematosus react exclusively with P1 and P2 and, in addition, with a third acidic protein designated P0 (19, 20). These three proteins also react with a mouse monoclonal antibody raised against chicken ribosomes (17).

The spatial arrangement of the P proteins in the eukaryotic ribosome is not yet well established. We have investigated the topography of P1 and P2 [designated eL12′ and eL12 in *Artemia* ribosomes (10)] by using the protein–protein crosslinking reagent 2-iminothiolane.

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MATERIALS AND METHODS

Materials. 2-Iminothiolane, dithiothreitol, and Coomassie blue R-250 were purchased from Pierce. Triethanolamine and 30% H_2O_2 were from Fisher. Acrylamide and *N,N′*-methylenebis(acrylamide) were from Kodak. Iodoacetamide and catalase were from Sigma; urea (ultrapure) and sucrose (ribonuclease-free) were from Schwarz/Mann. (^{125}I)Iodine (carrier-free, 17 Ci/mg; 1 Ci = 37 GBq) was purchased from New England Nuclear.

Preparation of *Artemia* 60S Ribosomal Subunits. Crude *Artemia* 80S ribosomes were prepared as described (21). The 60S subunits were isolated by centrifugation through 15–30% sucrose gradients in 0.3 M KCl/3 mM $MgCl_2$/0.5 mM dithiothreitol/20 mM Tris·HCl, pH 7.6, for 12 hr at 24,000 rpm in a Beckman SW27 rotor at 4°C.

Crosslinking of the 60S Subunits and Extraction of Proteins. The 60S subunits (50 A_{260} units/ml) in 4 mM $MgCl_2$/60 mM KCl/10 mM dithiothreitol/0.25 M sucrose/20 mM triethanolamine·HCl, pH 8.0, were treated with 4 mM 2-iminothiolane at 0°C for 50 min. The reaction was stopped by addition of 20 mM glycinamide. The modified subunits were incubated with 40 mM H_2O_2 at 0°C for 30 min to promote disulfide-bond formation, as described (22). Unreacted H_2O_2 was removed by incubation with catalase (10 μg/ml) for 15 min at 0°C. The unoxidized sulfhydryl groups were alkylated with 40 mM iodoacetamide to preclude possible disulfide interchange or random oxidation (23). The modified particles were collected by centrifugation at 40,000 rpm for 16 hr in a Beckman 60 Ti rotor. The ribosomal pellet obtained was suspended (about 400 A_{260} units/ml) in 1 mM $MgCl_2$/50 mM iodoacetamide/20 mM Tris·HCl, pH 7.6. Proteins were extracted by the addition of 1 volume of 8 M urea/6 M LiCl. This solution was adjusted to 66% (vol/vol) acetic acid to completely precipitate the RNA, as described (24). The proteins were collected by the addition of 8 volumes of cold acetone (24).

Fractionation of Crosslinked Acidic Proteins by Stepwise Elution from CM-Cellulose. The extracted total proteins (10–15 mg) in buffer A (6 M urea/3 mM iodoacetamide/20 mM Tris·HCl, pH 7.2) were loaded onto a column (1 × 4 cm) of CM-cellulose equilibrated with the same buffer. Proteins were eluted with buffer A, and then successively with buffer A containing 0.1 M, 0.2 M, and 0.5 M LiCl. Each fraction was dialyzed against 6% acetic acid at 4°C for 24 hr and then lyophilized.

Elution of the proteins from CM-cellulose at pH 5.0 (6 M urea/10 mM sodium acetate/0.1% methylamine/3 mM iodoacetamide, adjusted to pH 5.0 with acetic acid) instead of pH 7.2 was also used for some experiments.

Abbreviations: P proteins, eukaryotic ribosomal proteins P0, P1, and P2; NEPHGE, nonequilibrium pH gradient electrophoresis.

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Analysis of Crosslinked Proteins by "Diagonal" NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. Each protein fraction was dissolved in NaDodSO$_4$ sample buffer containing 40 mM iodoacetamide. Electrophoresis in the first dimension was performed under oxidizing conditions. The gel cylinder was removed, soaked in the reducing agent, and then embedded at the top of a gel slab for electrophoresis in the second dimension. The details of the procedures have been given previously (25–27).

Identification of Crosslinked Pairs Isolated from Diagonal Gels. Stained spots of each crosslinked pair that appeared to involve P proteins were cut out from the diagonal gel slab. Protein components were radioiodinated using chloramine T, as described (25, 28). The radiolabeled materials were mixed with total nonradioactive 60S proteins and submitted to two-dimensional gel electrophoresis in a system with acidic urea gel in the first dimension (pH 4.4) and NaDodSO$_4$ gel in the second dimension, as described previously (25). The gels were stained with Coomassie brilliant blue and dried for autoradiography.

Two-Dimensional Gel Analysis in Nonequilibrium pH Gradient–NaDodSO$_4$ System. The protein sample (≈70 μg) was dissolved in 9 M urea/2% (vol/vol) Nonidet P-40/2% (wt/vol) ampholytes (pH 3–10)/10 mM iodoacetamide and applied to a gel (2.5 × 110 mm) for nonequilibrium pH gradient electrophoresis (NEPHGE; ref. 29), as described previously (30), with the following modifications. Electrophoresis was performed at 200 V for 12 hr from the anode to the cathode. After electrophoresis in the first dimension, the gel was soaked for 2 hr in 2% NaDodSO$_4$/50 mM Tris·HCl, pH 6.8, and embedded on the top of a NaDodSO$_4$/polyacrylamide gel slab (125 mm wide, 100 mm long, and 1.5 mm thick). The gel slab contained a 16% acrylamide separation gel (95 mm long) with a 5% acrylamide stacking gel (5 mm long). Electrophoresis in the second dimension was performed at 150 V for 4 hr (31).

RESULTS

Fig. 1 shows a two-dimensional (acidic urea–NaDodSO$_4$ system) gel electrophoretic pattern of total proteins from *Artemia* 60S subunits. It has been reported (19, 20) that P proteins P0, P1, and P2 are the targets of anti-ribosome antibodies from systemic lupus erythematosus patients. We identified these P proteins on our two-dimensional gel by immunoblotting with lupus erythematosus anti-P-protein antibodies (kindly provided by N. Brot and K. Elkon) as designated in Fig. 1. Also, we observed that P1 and P2, but not P0, are extracted nearly quantitatively in ethanol/KCl solution (T.U. and R.R.T., unpublished data). P1 is typically observed as a faint spot compared to P2 on the gel of total 60S proteins. However, when P1 and P2 were selectively extracted with ethanol/KCl and then analyzed by one-dimensional NaDodSO$_4$/polyacrylamide gel electrophoresis, both P1 and P2 were stained to a similar extent by Coomassie blue (data not shown). The faint staining of P1 in two-dimensional gels of total protein may be due to its failure to enter the first-dimension gel because of its low pI value. The molecular weights of P1, P2, and P0 as determined by their mobility in the presence of NaDodSO$_4$ are 13,000, 12,000, and 34,000, respectively.

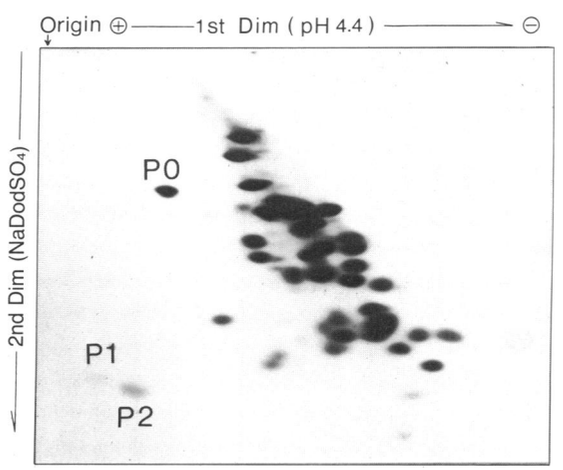

FIG. 1. Two-dimensional gel electrophoresis of total proteins from *Artemia* 60S ribosomal subunits. Electrophoresis in the first dimension was performed in 4% acrylamide/urea gel at pH 4.4 (24). The gel was soaked in 2% NaDodSO$_4$/50 mM Tris·HCl, pH 6.8, and submitted to electrophoresis in the second dimension (16% acrylamide). The locations of P1, P2, and P0 on the gel were determined by immunoblotting using lupus erythematosus anti-P-protein antibodies (18, 19).

Crosslinking of eukaryotic ribosomal subunits with 2-iminothiolane produces a large number of different protein dimers (25, 32, 33). Our attention here is focused on the crosslinks involving acidic proteins P1 and P2 formed in intact 60S subunits. Following crosslinking at 4 mM 2-iminothiolane, total protein was extracted from the 60S subunits and partially fractionated by stepwise elution from CM-cellulose at pH 7.2 (Fig. 2). The acidic proteins bind less tightly and are eluted early. The flow-through fraction (A), the fraction eluted by 0.1 M LiCl (B), and the fraction eluted by 0.2 M LiCl (C) were analyzed by diagonal gel electrophoresis (Fig. 3). Components arising from a single crosslink are visible below the diagonal and aligned on the same vertical line.

In the diagonal gel of fraction A, there are three vertical lines, indicated by arrows, that intersect one or more of the P proteins. These are designated A-1, A-2, and A-3. Line A-1 intersects P0 and P1; line A-2 intersects only P1; and line A-3, distinctly to the right of line A-2, intersects only P2. The proteins present in each spot were identified by cutting out the spots, radioiodinating the protein therein, and analyzing it by two-dimensional electrophoresis with nonradioactive total 60S protein as markers (Fig. 4). The two components on

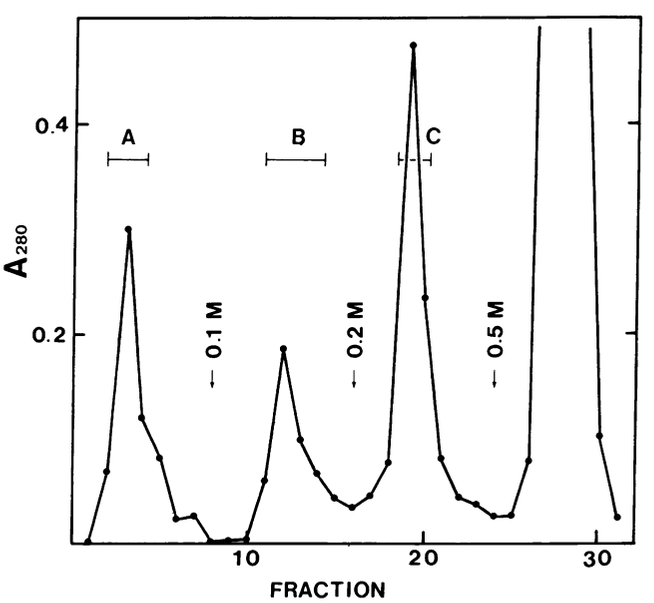

FIG. 2. Fractionation of crosslinked acidic proteins. Proteins extracted from crosslinked 60S subunits were eluted from a column of CM-cellulose by stepwise elution with LiCl. Flow-through fraction (A) and 0.1 M LiCl (B) and 0.2 M LiCl (C) fractions were pooled for further analysis.

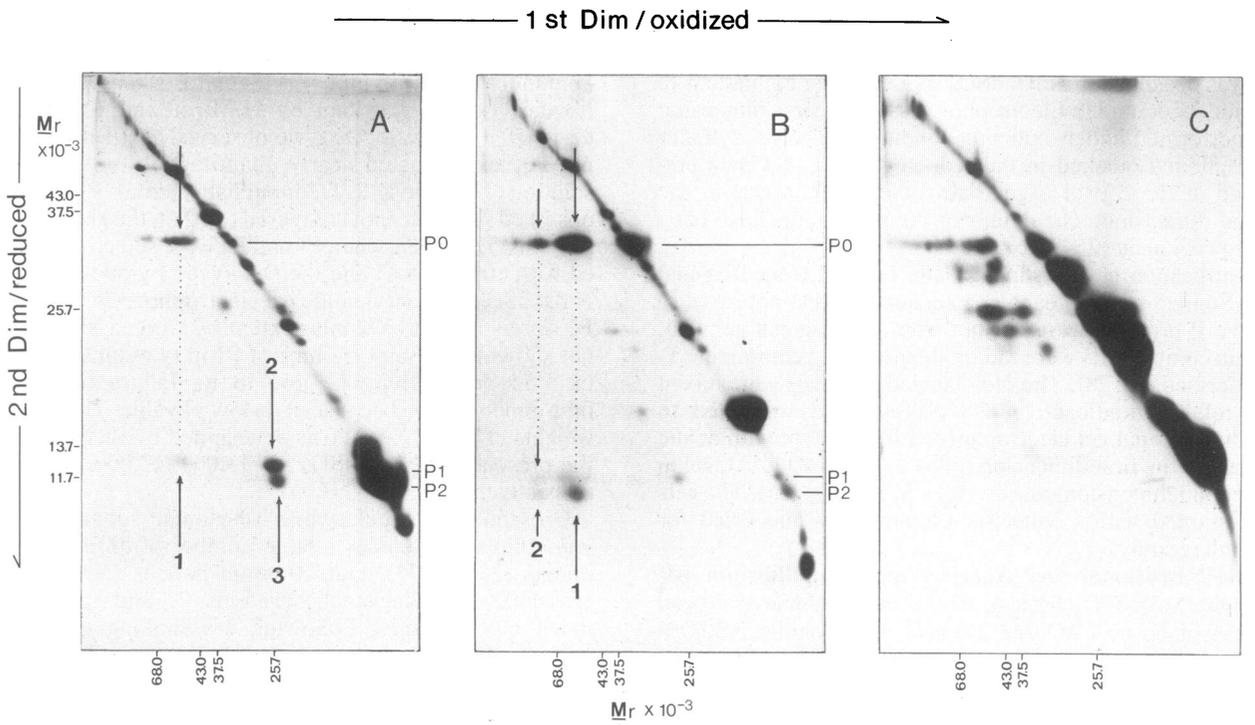

FIG. 3. "Diagonal" $NaDodSO_4$/polyacrylamide gel electrophoresis of protein fractions A–C (see Fig. 2) from CM-cellulose. Electrophoresis in the first dimension (13% acrylamide) was in the absence of reducing agents. After electrophoresis, the first-dimension gels were soaked in 3% 2-mercaptoethanol/2% $NaDodSO_4$/50 mM Tris·HCl, pH 8.8, for 15 min at 65°C and then in 0.1% $NaDodSO_4$/50 mM Tris·HCl, pH 6.8, for 30 min at room temperature. The gels were embedded at the top of gel slabs (16% acrylamide) for electrophoresis in the second dimension. The positions of monomeric P1, P2, and P0 on the diagonal are indicated. Numbers are given to individual pairs that appear to involve P1 and P2.

line A-1 are P0 and P1, the single A-2 spot contains only P1, and the single A-3 spot contains only P2. The results show the crosslink P0–P1 and the homodimers P1–P1 and P2–P2, respectively. The apparent molecular weights of the parent dimers obtained from calibration of the first dimension, and the sum of the molecular weights of the monomeric proteins from the second dimension, are consistent with this conclusion.

In the diagonal gel of fraction B (Fig. 3), there are two vertical lines that intersect P proteins. Line B-1 intersects P0 and P2 and line B-2 intersects P0, P1, and P2. The identity of the components on line B-1 was confirmed as described

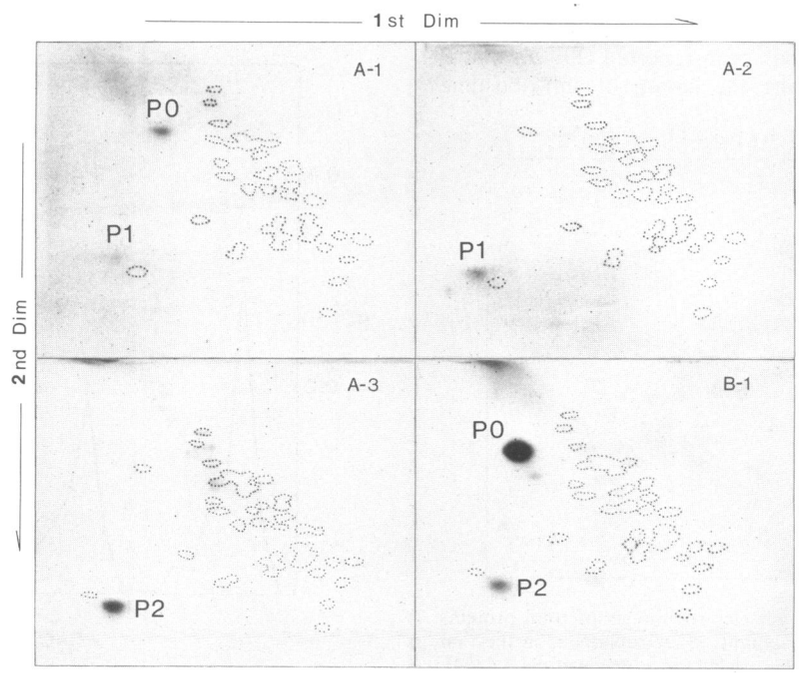

FIG. 4. Identification of crosslinked protein pairs. Protein components of crosslinked pairs (A-1, A-2, A-3, and B-1) were radioiodinated and submitted to two-dimensional (acidic urea–$NaDodSO_4$) gel electrophoresis with nonradioactive total 60S proteins (see Fig. 1). Proteins were stained with Coomassie blue. The gels were subjected to autoradiography. The positions of stained spots of carrier 60S proteins are indicated by dotted lines on the autoradiographs.

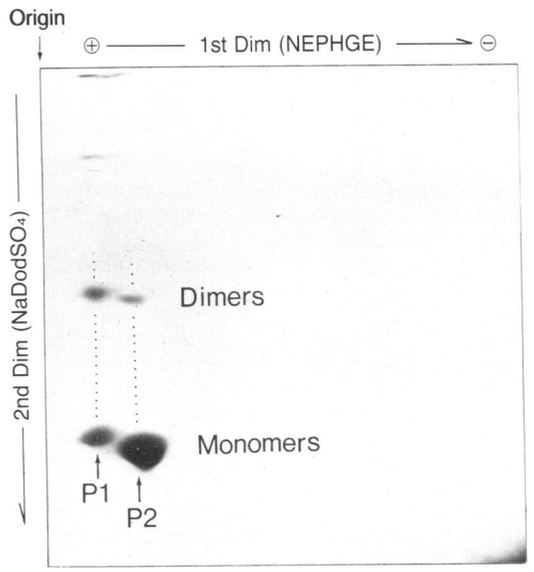

FIG. 5. Two-dimensional NEPHGE–NaDodSO$_4$ gel analysis of crosslinked acidic proteins. A protein fraction consisting of P1 and P2 was obtained from total crosslinked protein sample by elution from CM-cellulose at pH 5.0. Two-dimensional gel electrophoresis in NEPHGE–NaDodSO$_4$ system was performed under nonreducing conditions.

above and indicated the formation of a P0–P2 crosslink (Fig. 4). The apparent molecular weights are again consistent with this conclusion. The line B-2 is clearly shifted to the left from those representing the two dimers A-1 and B-1. The presence of all three P proteins on line B-2, which corresponds to an apparent molecular weight in the neighborhood of 70,000, indicates the formation of a multimer containing P0, P1, and P2. Such a trimer with one copy each of P1, P2, and P0 would have an actual molecular weight of 59,000; a tetramer with three copies of P1 and/or P2 plus one copy of P0 would have a molecular weight of 71,000–72,000. The accuracy of the gel system in this range is insufficient to distinguish unambiguously between these alternatives. There is a stained spot corresponding to P0 to the left of line B-2 (Fig. 3). This could represent the pentamer P0(P1)$_2$(P2)$_2$; however, the gel system does not give accurate values for these higher molecular weights.

In order to confirm the presence of homodimers P1–P1 and P2–P2, another two-dimensional gel analysis, in a NEPHGE–NaDodSO$_4$ system, was carried out. The acidic fraction was obtained from total crosslinked protein by elution from CM-cellulose at pH 5.0 instead of pH 7.2.

The proteins were analyzed by two-dimensional electrophoresis in the NEPHGE–NaDodSO$_4$ system under nonreducing conditions (Fig. 5). Two high molecular weight species of M_r 25,000 and M_r 24,000 were observed. The migration of each of these components in the first (NEPHGE) dimension was the same as that of monomeric P1 (M_r 13,000) and P2 (M_r 12,000), respectively. There was no indication of a spot midway between the P1 and P2 lines, the position expected for a P1–P2 heterodimer. When the electrophoresis was performed under reducing conditions, these high molecular weight species were not observed (data not shown). These results strongly suggest that P1 and P2 formed crosslinked homodimers but not heterodimers.

DISCUSSION

The acidic proteins of *E. coli* ribosomes have been the focus of extensive investigation because of their unique stoichiometry, four copies per 50S subunit (1), and their involvement in GTP-related processes in protein synthesis (6–8). Amino acid sequence analysis of acidic proteins from ribosomes of archaebacterial and eukaryotic organisms indicates structural similarity with the *E. coli* acidic proteins L7/L12 (12, 34, 35). Preliminary experiments suggest their presence in multiple copies and functional assays have pointed to their involvement in GTP-related processes (13–15). Our experiments were directed toward determining whether or not eukaryotic acidic ribosomal proteins are, like L7/L12, present in multiple copies, and the other ribosomal proteins to which they are proximal in the large ribosomal subunit.

In the work reported here, *Artemia* 60S ribosomal subunits were crosslinked with 2-iminothiolane to investigate interactions of the acidic proteins P1 and P2 present in this eukaryotic ribosome. In addition to P1 and P2, a third acidic protein, called P0 by some investigators and A_{33} by others, is found in eukaryotic ribosomes (17–20, 25). P0 has been shown to crossreact with a monoclonal antibody to P1 and P2 (17), and all three proteins react with the serum of certain patients with systemic lupus erythematosus (19, 20). Diagonal gel analysis of the acidic fraction of crosslinked 60S ribosomal proteins showed the proximity of this third protein P0 with the better studied P1 and P2, since crosslinks P0–P1 and P0–P2 were conspicuous. These gel analyses also suggested the presence of P1–P1 and P2–P2 homodimers. However, it is difficult to analyze crosslinks between P1 and P2 (three possibilities exist, P1–P1, P2–P2, and P1–P2) unambiguously by diagonal gel analysis, since the molecular weights of P1 (M_r 13,000) and P2 (M_r 12,000) do not differ enough for their crosslinked complexes to be well resolved on the diagonal gels. We therefore used another two-dimensional electrophoretic method, NEPHGE, followed by NaDodSO$_4$ gel electrophoresis. This gel system resolved P1 and P2 completely on the basis of their different isoelectric points. Two dimeric complexes with the same isoelectric points as the monomers were observed. One contained only P1 and the other only P2. There was no evidence for the existence of P1–P2 heterodimers. We conclude that P1 and P2 are present as separate dimeric complexes in the 60S subunit, each of which is anchored to the same protein, P0. The absence of P1–P2 heterodimers suggests that, despite their proximity to P0, P1 and P2 are not closely oriented in a manner to allow crosslinking.

An experiment was carried out with the P1/P2 mixture extracted selectively from the 60S subunit in ethanol/KCl solution. The extracted P1/P2 proteins were crosslinked with 2-iminothiolane and analyzed by the NEPHGE–NaDodSO$_4$ system (data not shown). Only homodimers P1–P1 and P2–P2 were observed. The results suggest that P1 and P2 exist as stable homodimers even in solution, like *E. coli* L7/L12 (36–38), and that there is no interaction of comparable stability between the two dimers. An earlier study (10) showed the presence of 1.8 copies of P2 in *Artemia* 80S ribosomes and its presence as a dimer in solution; however, only 0.9 copy of P1 was observed.

The results summarized above do not reveal whether or not a single ribosome contains both P1 and P2. From these results alone, heterogeneity could not be ruled out and it would be possible that some ribosomes had only P1 and others only P2. However, the finding of a trimer that contained P0, P1, and P2 necessitates the simultaneous presence of all three proteins in the same ribosomal particle. This result argues for the simplest explanation, that the ribosome population is homogeneous.

Fig. 6 shows the arrangement of the P proteins in *Artemia* 60S subunit deduced from crosslinking results. The P proteins are represented as a pentameric complex (P1)$_2$(P2)$_2$P0. This model is compared to that for *E. coli* in which the two L7/L12 dimers are anchored by L10 (3, 4). The *E. coli* model represents recent evidence for a bent conformation for one of the dimers (9). Protein P0 in eukaryotes appears to play the

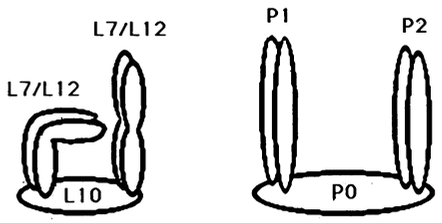

FIG. 6. Possible arrangement of acidic proteins in *E. coli* (*Left*) and *Artemia* (*Right*) large ribosomal subunits.

same role as L10 in *E. coli*, as suggested recently by Elkon *et al.* (18). The homology between P0 and the other conserved acidic proteins P1 and P2 appears unique: there is no obvious homology between L10 and L7/L12 (39), nor immunological crossreactivity (ref. 40; B. Nag and R.R.T., unpublished observation).

Our results are consistent with previous crosslinking studies with rat liver ribosomes (24, 25). Protein A_{33}, which has been found to correspond to P0 by immunoblotting with lupus erythematosus anti-P-protein antibodies (T.U. and R.R.T., unpublished data), was crosslinked to rat liver P1 with 2-iminothiolane. Furthermore, both of the proteins A_{33} (P0) and P2 were crosslinked to elongation factor EF-2 (24), a finding that places P2, as well as P1, in the same general neighborhood with P0. A more detailed study of the rat liver acidic protein crosslinks has not been performed. The results from *Artemia* and rat liver suggest that the presence of ribosomal acidic proteins in a pentameric complex may be a common organizational feature in ribosomes.

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