Acidic Proteins of the Large Ribosomal Subunit in *Saccharomyces cerevisiae*. Effect of Phosphorylation†

F. Juan Vidales, M. T. Saenz Robles, and J. P. G. Ballesta*

ABSTRACT: Three strongly acidic proteins with p*I*s between 3.0 and 3.5 have been detected and purified from an ammonium–ethanol extract of *Saccharomyces cerevisiae* ribosomes. The three proteins, called L45, L44, and L44′, have a similar amino acid composition, but differences were shown by tryptic peptide analysis. Nevertheless, the three polypeptides show total cross-reaction to antisera raised against one of them. Protein L44′ is very unstable in the extract when treated at the basic pH 9.2, due to an enzymatic process not yet clarified. When purified, the protein is, however, stable. In solution, the proteins are present as dimers, as verified by ultracentrifugation, column filtration, and photochemical cross-linking. The tendency to dimerization is much lower in the case of protein L44′. On the average, 3.2 copies of these proteins are detected per ribosome. The proteins are monophosphorylated when present in the ribosome. Phosphorylation seems to regulate the affinity of the polypeptides for the particles because unphosphorylated proteins bind poorly to the ribosomes deprived of the acidic proteins. Since these proteins are unphosphorylated when present in the cytoplasm [Zinker, S. (1980) *Biochim. Biophys. Acta 606*, 76–82; Sánchez-Madrid, F., Vidales, F. J., & Ballesta, J. P. G. (1981) *Eur. J. Biochem. 114*, 609–613], a regulatory mechanism of the ribosomal function based on a phosphorylation–dephosphorylation process of the acidic proteins is being studied.

The strongly acidic proteins of the large ribosomal subunit are probably among the most interesting components of the ribosome structure. These proteins, initially characterized in the bacterial ribosome and named according to the international nomenclature as L7 and L12 (Kaltschmidt & Wittman, 1970), turned out to be a unique polypeptide with its amino terminal either free (L12) or blocked (L7). The four copies, present in the particle as a pair of dimers, form a complex with protein L10 and seem to constitute one of the three protuberances detected in the large subparticle by electron microscopy [for a review, see Liljas (1982)]. These proteins have an important role in the interaction of the supernatant factors with the ribosome. However, the data so far available seem to indicate that the proteins do not form the binding site for the factors (Maasen & Möller, 1981), and the particles can carry out some of the elongation factor functions in their absence (Ballesta & Vázquez, 1972; Hamel & Nakamoto, 1972). They probably induce the appropriate particle conformation for the interaction to take place under the proper conditions. In fact, changes in the ribosomal structure have been detected in the presence of L7 and L12 (Michalski & Sells, 1975).

Confirming the importance of these proteins in ribosome function is the ubiquitous presence of similar proteins in all the species so far studied (Vidales et al., 1981a). Proteins with p*I*s lower than L7 and L12 have been reported in rat liver (Stöffler et al., 1974; Reyes et al., 1977), *Artemia salina* (Möller et al., 1975), yeast (Kruiswijk & Planta, 1975; Zinker & Warner, 1976; Sánchez-Madrid et al., 1979a; Itoh et al., 1979), Krebs II ascites cells (Leader & Coia, 1977), HeLa cells (Horak & Schiffman, 1977), *Drosophila melanogaster* (Chooi et al., 1980), rabbit reticulocytes (Howard et al., 1975), wheat germ (Madrzak et al., 1979), mouse myeloma cells (Martini & Kruppa, 1979), *Tetrahymena piriformis* (Sandermann et al., 1979), and chicken reticulocytes (Howard et al., 1976). In some cases, the proteins have been characterized (van Agthoven et al., 1978; Tsurugi et al., 1978) and even sequenced (Itoh, 1981; Lin et al., 1982).

We have selected the yeast *Saccharomyces cerevisiae* as an appropriate system to carry out studies on the ribosomal acidic proteins. We have previously reported the existence of two acidic proteins (Sánchez-Madrid et al., 1979a; Vidales et al., 1981b) and also that this system seems to be less dependent than others on the presence of these proteins for some ribosomal activities, although the stimulatory effect of the acidic proteins in the functions of elongation was evident (Sánchez-Madrid et al., 1979b). The equivalence of the yeast proteins to the bacterial L7 and L12 was also shown in a hybrid system where the *Escherichia coli* proteins were able to reconstitute the yeast ribosome functions (Wool & Stöffer, 1974; Richter & Möller, 1974; Sánchez-Madrid et al., 1981a).

Contrary to other systems, and even to other ribosomal proteins in yeast, these proteins are only monophosphorylated and appear unphosphorylated in the cytoplasm (Zinker, 1980; Sánchez-Madrid et al., 1981b). In rat liver, phosphorylation of the acidic proteins seems to affect the activity of the particles (MacConmell & Kaplan, 1982) although the opposite results have been reported in ascites cells (Leader et al., 1981). We present here data indicating that phosphorylation might control the affinity of the acidic proteins for the ribosome in yeast, as was previously shown for other nonribosomal proteins (Sánchez-Madrid et al., 1981b). These data indicate that in eukaryotic ribosomes, phosphorylation adds a new aspect to the role of these interesting proteins in the control of the ribosome functions. In any case, to understand this role, the different proteins and the mechanisms regulating the presence of the different forms of acidic proteins in the ribosome must first be characterized. As a first step, we report here the isolation and physicochemical characterization of the three proteins present in the ribosome of *Saccharomyces cerevisiae*.

Materials and Methods

Organisms and Growth Conditions. Saccharomyces cerevisiae Y166 was used throughout the work presented here.

† From the Centro de Biología Molecular, CSIC and UAM, Canto Blanco, Madrid 34, Spain. *Received March 10, 1983; revised manuscript received July 29, 1983.* This work has been supported by institutional grants from the Fondo de Investigaciones Sanitarias (FIS) and the Dirección General de Sanidad and by a personal grant to J.P.G.B. from the Comisión Asesora de Investigación Científica y Técnica (Spain).

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Cells were grown in yeast extract–peptone–glucose (YEP) medium in a 30-L New Brunswick fermentor up to mid-exponential phase, at 30 °C. When ^{32}P-labeled ribosomes were required, growth was carried out in a 2-L flask with 1 L of phosphate-free rich medium (Rubin, 1973) in the presence of 10 mCi of $^{32}PO_4^{3-}$ for at least three doubling times at 30 °C, up to A_{660} = 1.0.

Ribosomes were prepared from sea sand ground cells as described (Sánchez-Madrid et al., 1979a,b). Proteins were extracted from the ribosomes by washing with 50% ethanol in the presence of 1 or 0.5 M NH_4Cl to obtain the split protein fraction $SP_{1.0}$ and the corresponding $P_{1.0}$ core particles in the first case and the $SP_{0.5}$ fraction and $P_{0.5}$ cores in the second case.

The proteins were precipitated from the extract with 2.25 volumes of acetone at −25 °C and stored at −80 °C. The core particles were dialyzed against 20 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl),[1] 8 mM $MgCl_2$, 50 mM KCl, and 10 mM β-mercaptoethanol and stored at −80 °C.

Chromatographic Procedures. Purification of the proteins was carried out as described previously (Vidales et al., 1981b), except that in our case the proteins were treated with alkaline phosphatase before chromatography through CM-cellulose and DEAE-cellulose. $SP_{0.5}$ proteins were extracted from exponentially growing ribosomes in order to minimize the content of protein Ax. By chromatography in DEAE-cellulose, using a gradient from 50 mM ammonium acetate, pH 5.7, to 1 M ammonium acetate, pH 4.6, in 6 M urea, we separated protein L45 from proteins L44 and L44′. The eluted fractions from the DEAE-cellulose column were followed by electrofocusing. The fractions containing L44 and L44′ were dialyzed against 5% acetic acid, lyophilized, dissolved in 25 mM $NaHCO_3$, pH 8.0, and 1 M NaCl, and passed through a Sephadex G-100 filtration column. The fractions were also tested by electrofocusing, and those containing pure proteins were dialyzed against 5% acetic acid and lyophilized.

Alkaline Phosphatase Treatments. Proteins in 100 mM Tris-HCl, pH 7.4, and 10 mM β-mercaptoethanol were incubated with alkaline phosphatase (Sigma Chemical Co.) either soluble or attached to Sepharose (1 enzyme unit per 200 μg of protein), at 37 °C for 2 h. The mixture, after centrifugation in the case of the insoluble enzyme, was lyophilized and dissolved in the appropiate buffer for chromatography or electrophoresis.

Amino Acid Analysis. Proteins were hydrolyzed under vacuum with 6 N HCl in the presence of 0.02% β-mercaptoethanol in sealed ampules at 110 °C for 20 h. The hydrolysates were dried under vacuum and dissolved for analysis in a Beckman 121 amino acid analyzer.

Tryptic Digestion. Proteins (100 μg) were treated with trypsin (1 μg) in 0.2 M ammonium bicarbonate, pH 8.5, for 4 h at 37 °C. After a double-lyophilizing process, the samples were resolved in 20 × 20 × 0.1 cm cellulose plates in two dimensions: first, by electrophoresis in 4% pyridine and 1.25% acetic acid, pH 5.4, for 2 h at 400 V; second, by ascending chromatography in pyridine, acetic acid, water, and 1-butanol (10:3:15:12). The spots were detected by ninhydrin in 1% acetone.

Electrophoretical Techniques. Electrophoresis was carried out in the presence of sodium dodecyl sulfate (SDS) according to Laemmli & Favre (1973). Electrofocusing was performed in 5% acrylamide gel slabs (150 × 130 × 2 mm) by using 2% Pharmacia ampholytes, pH 2.5–5.0, over a water-refrigerated plate, in the cold room, and applying a constant current of 6 mA until the voltage reached 600 V and then a constant voltage of 250 V for 14 h. The gels were stained with Coomassie blue in 12% trichloroacetic acid (Blakesley & Boezi, 1977).

Ultracentrifugation studies were performed in a Beckman E ultracentrifuge. To determine the sedimentation coefficient, a solution of proteins with 5 mg/mL in 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 60 mM NH_4Cl, and 2 mM β-mercaptoethanol was run at 56000 rpm at 20 °C. The molecular weight was determined by using protein concentrations of 0.3, 0.5, and 0.8 mg/mL in the same buffer, run at 34000 and 40000 rpm at 12 °C (Yphantis, 1964).

Immunochemical Techniques. Antisera against proteins were obtained from rabbits by intramuscular injection of about 0.2 mg of protein in 1 mL of complete Freund's adjuvant, followed by several subcutaneous booster injections of 1 mg in incomplete adjuvant every 15 days.

Radial immunodiffusion was performed in 1.5% agar plates containing 0.1–0.2 mL of serum per mL of gel in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl. The sample of protein dissolved in the same buffer was placed in wells of 3 mm id and allowed to diffuse for 18 h at room temperature.

Radioactive Labeling of Proteins. Iodination was carried out by using chloramine T according to a method that avoids damage of the sample, by keeping the substrate and the oxidant agent in a separate phase (Tejedor & Ballesta, 1982). ^{125}I (15 mCi/μg) from Amersham International was used.

Phosphorylated and unphosphorylated proteins were treated under identical conditions.

Binding of Proteins to Ribosomal Particles. Three hundred picomoles of particles in 50 mM Tris-HCl, pH 7.4, 8 mM $MgCl_2$, 60 mM KCl, and 10 mM β-mercaptoethanol was incubated at 37 °C for 30 min with a 10-fold excess of either phosphorylated or unphosphorylated acidic proteins. The final specific activity of acidic proteins in the mixture was 95 cpm/pmol. After incubation, the samples were passed through a Sepharose 6B column and eluted by using the same incubation buffer. Fractions were taken and the radioactivity and A_{260} monitored in each fraction.

Photochemical Cross-Linking. Proteins at 0.2 mg/mL were treated with 15 and 75 μg of ethyl 5-(2-methoxy-4-nitrophenoxy)valerimidate (1 mg/mL) (a gift of Dr. F. Tejedor) at 20 °C for 6 h, in 50 mM $NaHCO_3$, pH 9.2, and 20% methanol. After incubation, the samples were treated with the same volume of ethyl acetate to extract the unreacted cross-linker. They were then lyophilized, taken into 50 mM barbital, pH 8.0, and irradiated at 5 cm for 3 h at 4 °C by using a Sylvania F8T5/BLB lamp. After this, the sample was dialyzed against water, lyophilized, and dissolved in sample buffer for electrophoresis.

Activity Tests. Elongation factor 2 (EF-2) dependent GDP binding was carried out in a 50-μL mixture containing 10 pmol of ribosomes in 50 mM Tris-HCl, pH 7.4, 60 mM KCl, 8 mM $MgCl_2$, and 5 mM β-mercaptoethanol. [^3H]GDP concentration was 7 μM. A partially purified preparation of EF-2 from reticulocytes was used at 0.5 mg/mL final concentration. The reaction was carried out as described elsewhere (Sánchez-Madrid et al., 1979b).

Results

Acidic Ribosomal Proteins in the S. cerevisiae Ribosome. Proteins extracted by ammonium chloride–ethanol (Sánchez-Madrid et al., 1979b) from the *S. cerevisiae* ribo-

[1] Abbreviations: PMFS, phenylmethanesulfonyl fluoride; Tris, tris-(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

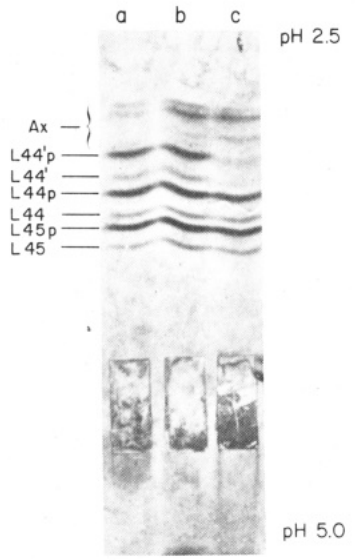

FIGURE 1: Electrofocusing of *S. cerevisiae* acidic proteins. Proteins extracted from ribosomes by 50% ethanol–0.5 M ammonium chloride were either dissolved in 8 M urea and kept at 4 °C (a), dissolved in 8 M urea and 50 mM β-mercaptoethanol and incubated at 37 °C for 2 h (b), or dissolved in 25 mM $NaHCO_3$, pH 9.2, and 10 mM β-mercaptoethanol and incubated at 37 °C for 2 h (c). Electrofocusing was run as described under Materials and Methods between pH 2.5 (top) and 5.0 (bottom).

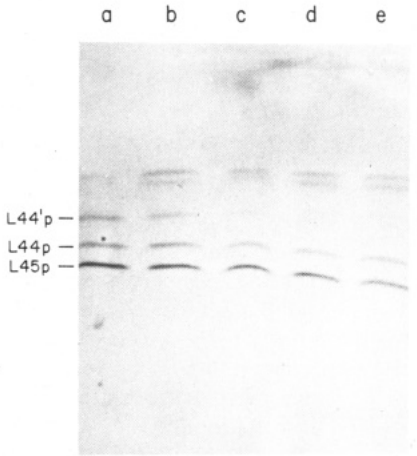

FIGURE 2: Acidic proteins ($SP_{0.5}$ fraction) incubated in 10 mM $NaHCO_3$, pH 9.2, at 37 °C in the presence of 30 mM sodium iodoacetate for 5 (b) and 30 min (d) and with 9 mM PMFS for 5 (c) and 30 min (a). The control sample was taken directly from the stock $SP_{0.5}$ fraction without carbonate treatment. Electrofocusing was as described in Figure 1.

some and analyzed by isoelectrofocusing in acrylamide or agarose gels show 8–10 bands, with p*I*s ranging from 3.0 to 4.0 (Figure 1). The four lower bands had been previously characterized as the phosphorylated and unphosphorylated forms of proteins L45 and L44 (Sánchez-Madrid et al., 1979), originally identified by two-dimensional gel electrophoresis as the possible counterparts of the bacterial proteins L7 and L12 (Kruiswijk & Planta, 1975; Zinker & Warner, 1977). the upper bands probably correspond to a nonribosomal protein, protein Ax, susceptible to multiphosphorylation and described elsewhere (Sánchez-Madrid & Ballesta, 1979). The protein in the intermediate position, called L44′, had not been detected previously, probably due to its high susceptibility to basic pH.

When the extracted proteins are dissolved in bicarbonate buffers at pH 9.2, protein L44′ disappears from the gels (Figure 1). The disappearance of L44′ appears to be mediated by the presence of some contamination in the extract, since

Table I: Phosphate Content of Acidic Ribosomal Proteins from *S. cerevisiae*[a]

protein	absorbance	cpm	cpm/ absorbance
$L44'_p$	16.9	1640	97
L44′	2.2	31	14
$L44_p$	19.5	1927	99
L44	2.3	22	10
$L45_p$	15.8	1423	90
L45	1.6		

[a] Acidic ribosomal proteins extracted from *S. cerevisiae* cells grown in the presence of $^{32}PO_4^{3-}$ by ammonium–ethanol washing were separated by isoelectrofocusing. The stained gel was scanned and the intensity of the bands estimated from the area of the peaks. Radioactivity was directly measured in the cut spots.

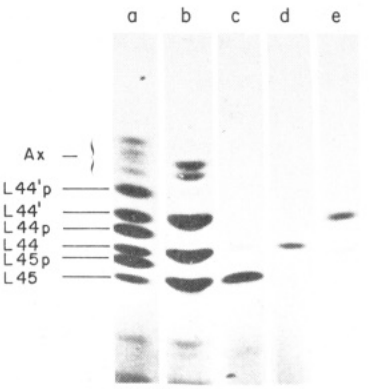

FIGURE 3: Electrofocusing of purified acidic ribosomal proteins: (a) mixture of acidic proteins used as the standard; (b) extracted acidic proteins after alkaline phosphatase treatment; (c) protein L45; (d) protein L44; (e) protein L44′.

protein L44′, when purified, is stable at basic pH. Its sensitivity to basic pH could suggest the action of a protease; however, if this is the case, the enzyme is insensitive to iodoacetic acid and PMFS (Figure 2).

A possible interconversion of the different proteins cannot be excluded, since, in some cases, an increase in the intensity of protein L45 or in one of the bands in the region of protein Ax has been detected (Figure 2). However, in most cases, the intensity of the remaining bands is not altered when protein L44′ disappears.

Phosphorylation of Acidic Proteins. Proteins L45 and L44 are present in the ribosome as monophosphorylated polypeptides (Sánchez-Madrid et al., 1981), and the fact that only one dephosphorylation band is detected below the position of L44′ (Figure 1) suggests that this is probably also the case for this last protein. Table I confirms this suggestion, showing that the ratio of band intensity to radioactivity, when cells are grown in the presence of $^{32}PO_4^{3-}$, is the same for the three proteins.

Purification of Acidic Proteins. By column chromatography using CM-Cellulose, DEAE-cellulose, and filtration through Sephadex G-100, we have been able to purify the unphosphorylated forms of the three proteins. The acidic proteins were prepared from exponential-phase ribosomes in order to minimize the content of protein Ax (Sánchez-Madrid & Ballesta, 1979). DEAE-cellulose chromatography of phosphatase-treated proteins, previously passed through CM-cellulose to remove the basic proteins extracted in minor quantities from the ribosomes, separates protein L45 from L44 and L44′ (not shown). Filtration through Sephadex G-100 allows the separation of proteins L44 and L44′. In this way, proteins containing less than 5% of other acidic proteins, as tested by electrofocusing (Figure 3), are obtained. The three proteins

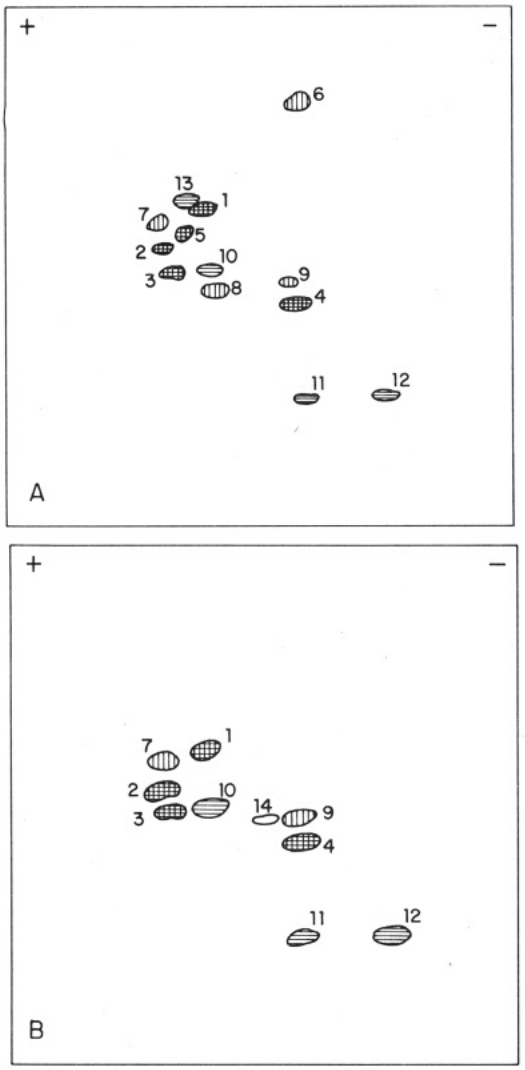

FIGURE 4: Tryptic digestion map of acidic proteins. (A) Peptide map of proteins L44 and L45. Spots with horizontal lines correspond to protein L45; those with vertical lines correspond to protein L44. (B) Peptide map of protein L44′. Spots with horizontal lines are also present in protein L45. Spots with vertical lines are also in protein L44. The empty spot is present only in protein L44′.

Table II: Amino Acid Composition of Proteins L45, L44, and L44′

amino acid	mol % L44′	L44	L45
Asp + Asn	12.9	10.4	8.1
Thr	2.2	2.4	2.0
Ser	4.8	5.5	5.5
Glu + Gln	14.4	16.2	12.4
Pro	0.7	2.5	2.3
Gly	13.4	10.0	13.4
Ala	24.3	21.1	24.2
Val	3.9	4.9	5.5
Met	1.0	0.9	1.0
Ile	3.3	4.5	3.8
Leu	8.0	9.9	9.5
Tyr	1.3	1.6	1.6
Phe	2.5	2.2	3.0
Lys	6.5	7.6	7.1
His	0.5		
Arg			0.8

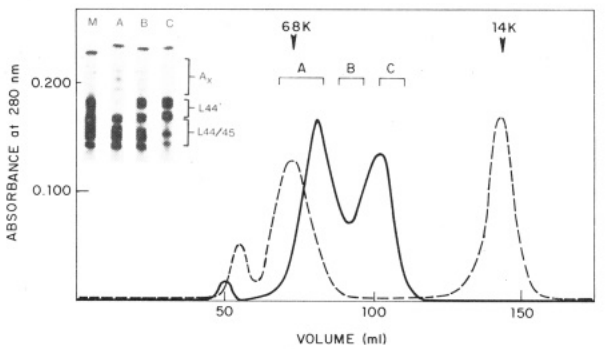

FIGURE 5: Filtration through Sephadex G-100 of acidic proteins of *S. cerevisiae*. The acidic proteins (2 mg) dissolved in 25 mM $NaHCO_3$, pH 8.0, and 1.0 M NaCl were applied to a 1 × 12 cm column of Sephadex G-100 and eluted with the same buffer. The absorbance was monitored at 280 nm, and aliquots of the section marked were pooled. Electrofocusing of the proteins in the pooled fractions and in the original sample (M) is shown in the inset. The discontinuous line indicates the position of the markers (bovine serum albumin, 68K; lysozyme, 14K). When the acidic proteins were filtered by using buffers of low ionic concentration (80 mM NaCl), the three proteins eluted at the position of peak A.

were indistinguishable by SDS gel electrophoresis, showing a similar molecular weight of around 13 500.

Amino Acid Composition. After hydrolysis with 6 M HCl, the composition of the three proteins was checked. Table II summarizes the results obtained for L45, L44, and L44′. In general, the amino acid compositions of the three proteins are quite similar. However, several significant differences can be detected among them. Arginine is present only in protein L45 and histidine in L44′; proline is drastically diminished in L44′. The content of acidic residues is highest in L44′ and lowest in L45, in agreement with their relative p*I*s.

Tryptic Digestion. Analysis of the peptides after tryptic digestion of the three unphosphorylated proteins results in patterns which are not exactly the same (Figure 4). Proteins L44 and L45 show 9 peptides each, and L44′ shows 10. Only four peptides are common to the three polypeptides (1, 2, 3, and 4). Seven peptides (1, 2, 3, 4, 10, 11, and 12) are present in L45 and L44′, six are in L44 and L44′ (1, 2, 3, 4, 7, and 9), and five (1, 2, 3, 4, and 5) are in L44 and L45. In principle, protein L44′ seems closer to L44 and L45 than these two proteins to each other.

Dimer Formation. When a mixture of the three acidic proteins is filtered through a Sephadex G-100 column at low ionic strength, the protein comes out of the column at a position corresponding to a molecular weight much higher than that expected, considering the values calculated from electrophoresis in SDS. This suggests that, like other acidic proteins from different ribosome species (Möller et al., 1972; Osterberg et al., 1976; van Agthoven et al., 1978), these proteins are present as aggregates, mostly dimers, in solution. Protein L44′ seems to form less stable aggregates since when the ionic concentration is increased this protein elutes in a retarded position (Figure 5). In both cases, however, the molecular weight estimated from the filtration data is considerably higher than that estimated from SDS (13 500) and ultracentrifugation (see below), indicating that the structure of these proteins differs from the globular model or, alternatively, that in the filtration conditions they form aggregates larger than dimers.

Ultracentrifugation analysis of the proteins also confirms their dimer character when in solution. Proteins L44 and L45 have a sedimentation coefficient value of 1.78 S and a molecular weight of 20 443, slightly lower than that expected for the dimer from the SDS gel electrophoresis data. Protein L44′ behaves as a monomer in this technique, giving an *s* value of 1.34 S and a molecular weight of 11 092.

Photochemical cross-linking using 5-(2-methoxy-4-nitrophenoxy)valerimidate also shows the presence of dimers and

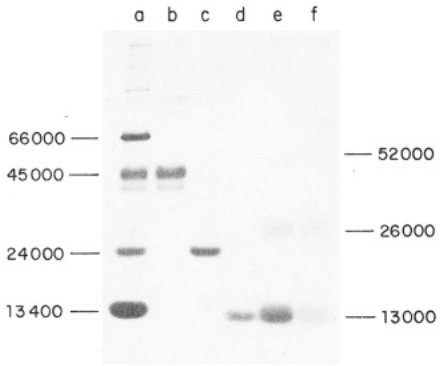

FIGURE 6: SDS gel electrophoresis of cross-linked acidic proteins. Phosphorylated acidic proteins (fraction $SP_{0.5}$) were treated with nitroguaiacyl butyrimidate at a molar ratio of reagent to protein of 1:1 (sample e) and 5:1 (sample f). Sample d corresponds to untreated proteins, and samples a–c correspond to molecular weight markers.

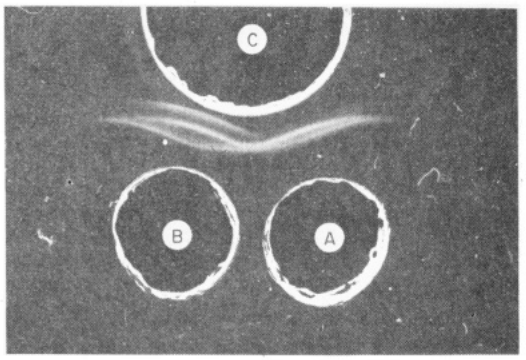

FIGURE 7: Immunodiffusion test of proteins L44′ and L44–L45: (A) 5 μg of proteins L44–L45; (B) 5 μL of proteins L44′; (C) anti-L45 serum.

even tetramers of acidic proteins in solution (Figure 6). The proteins were first modified chemically and afterward irradiated at 340 nm to induce covalent bond formation through the *p*-nitroguaiacyl residue (Jelenc et al., 1978). After irradiation, the samples were analyzed by SDS–polyacrylamide gel electrophoresis. When modification was carried out in the presence of a large excess of the chemical reagent (Figure 6, sample f), precipitation of proteins takes place in the sample, reducing the amount of material entering the gel.

Immunochemical Cross-Reaction. Antisera raised against protein L45 were tested with the three proteins by the double-immunodiffusion technique. Figure 7 shows that a mixture of proteins L45 and L44, previously found to be immunologically similar (Vidales et al., 1981b), gives a reaction totally identical with protein L44′. It is interesting to note that the intensity of the precipitation bands is different in the two samples. Since the various bands probably correspond to different aggregates of the proteins that diffuse at different speeds into the agarose, the band closer to the antiserum well must correspond to the monomers. This band is practically undetectable in L44 and L45 and very strong in L44′, confirming the lower tendency of this protein to dimerize in solution.

Using the same antisera, we have calculated the number of copies per ribosome of the proteins L45, L44, and L44′ by radial immunodiffusion, assuming a molecular weight of 13 500, using as standard a mixture of purified acidic proteins. In six determinations, we have obtained an average value of 3.2 copies per ribosome, although substantial differences are detected among different preparations; we have found samples with as low as 2.5 or as high as 3.7 copies per ribosome. At present, we cannot say whether the differences are due to losses

FIGURE 8: Electrofocused gels of $SP_{0.5}$ proteins used in reconstitution experiments before (A) and after (B) phosphatase treatment. Electrofocusing was performed as described in Figure 1.

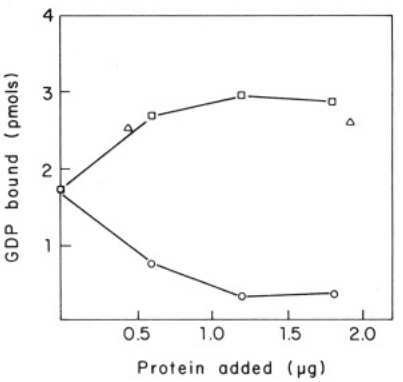

FIGURE 9: Reconstitution of EF-2-dependent binding of GDP to core particles. [^{3}H]GDP binding was performed as indicated under Materials and Methods in the presence of untreated (□) and phosphatase-treated (○) $SP_{0.5}$ proteins. Phosphorylated proteins, iodinated as described, were also tested under similar conditions (△); 3.22 pmol of GDP was bound by the control 80S ribosomes.

of proteins during the ribosome preparation or whether they reflect the actual content of these proteins on the ribosomes in the cells. We are now investigating the acidic protein content of the ribosomes during the cell cycle and metabolic alterations.

Function of the Ribosomal Acidic Proteins. The split proteins are able to restore the activity of the ribosomal core particles (Sánchez-Madrid et al., 1979b). The purified proteins, however, show very low reconstituting activity as compared to the original split fraction (not shown). Since purified proteins are unphosphorylated while those in the $SP_{0.5}$ fraction are phosphorylated (Figure 2), these results suggest that phosphorylation might be required for activity. This possibility was tested by using phosphorylated and phosphatase-treated $SP_{0.5}$ proteins (Figure 8 shows that phosphatase treatment causes total dephosphorylation of proteins) in the reconstituting assay. As shown in Figure 9, phosphorylated proteins are able to reconstitute the nucleotide binding capacity of the core particles while unphosphorylated proteins are inactive, producing, in fact, inhibition of the core's residual activity at high concentration.

The lack of reconstituting activity of unphosphorylated proteins might be due to an alteration of the affinity of the acidic proteins for the ribosome. Therefore, we have checked the binding properties of phosphorylated and unphosphorylated proteins. Proteins were labeled by iodination, a modification that does not affect their reconstituting activity (Figure 9). As shown in Table III, the unphosphorylated form of proteins binds poorly, while almost four copies of phosphorylated

Table III: Binding of Acidic Proteins to Ribosomal Particles[a]

particle	binding of unphosphorylated proteins (pmol of proteins/ pmol of ribosomes)	binding of phosphorylated proteins (pmol of proteins/ pmol of ribosomes)
80S ribosomes	0.84	0.46
$P_{0,5}$ cores	0.94	3.70

[a] Untreated and alkaline phosphatase treated $SP_{0,5}$ proteins were subjected to mild iodination under identical conditions, as indicated under Materials and Methods. After purification, they were mixed with unlabeled carrier acidic proteins to obtain the appropriate concentration and specific activity. The radioactive proteins were incubated with 80S ribosomes and $P_{0,5}$ core particles as described. Bound copies of proteins were calculated from the known specific activity.

proteins are bound per ribosomal core particle.

It is interesting to note that the unphosphorylated proteins have a higher tendency to bind unspecifically to the particles as indicated by the amount of protein bound to intact 80S ribosomes. This unspecific binding is probably responsible for the inhibition caused by the unphosphorylated proteins in the reconstitution assay (Figure 9).

These results strongly suggest that phosphorylation affects the conformation of the acidic proteins in such a way that the interaction with the ribosomal binding site is partially hindered. It is very likely that this change does, in fact, take place, since the susceptibility to iodination is also drastically diminished by phosphorylation: while a specific activity of 99.0 nCi/mg was obtained in the phosphorylated proteins, no more than 7.7 nCi/mg was reached in the unphosphorylated species when iodination was carried out under identical conditions.

Discussion

In bacteria, the presence of four copies of the acidic proteins in the ribosome, forming a pair of dimers, has been established (Liljas, 1982). The functional significance of this is still obscure, although there are some indications of a different role for each dimer, one of them perhaps implicated more directly in the binding of GTP and the other in its hydrolysis (Sánchez-Madrid et al., 1981). In some cases, only one dimer seems to be required for activity (Lee et al., 1981). Our data show that in *S. cerevisiae*, values close to four copies of acidic proteins per ribosome are obtained in some ribosomal preparations, and since these acidic proteins associate in solution, it is reasonable to suppose that they also bind to the ribosome as a pair of dimers. However, ribosome preparations with considerably fewer acidic ribosomal proteins have been obtained. The significance of these data is not clear, and although some kind of preparation artifact cannot be totally ruled out, we tend to believe that these results have, rather, a metabolic significance, related perhaps to the level of phosphorylation of the acidic proteins, as will be discussed below.

Three acidic ribosomal proteins, L44′, L44, and L45, are found in *S. cerevisiae* by isoelectrofocusing. One of them, protein L44′, described here for the first time, is very labile at basic pH. The three proteins are similar in their overall amino acid composition, although their tryptic digestion maps show clear differences. Itoh (1981) has published the amino acid sequence of one acidic ribosomal protein of *S. cerevisiae*. According to the data of Itoh (1981), this protein, initially called YPA1 and more recently YL44c (Otaka & Osawa, 1981), is the most acidic of them. If that is so, this protein would correspond to our L44′. However, its amino acid composition (Itoh, 1981) fits that of L45, the least acidic protein in our system, better. The final decision as to which one of our proteins corresponds to YPA1 cannot be made without further information, not yet available.

The relationship among proteins L45, L44, and L44′ is not clear at present. L44′ could be related to L45 since at least 7 out of 10 peptides move to similar positions in the tryptic map. However, L44′ lacks arginine and has considerably less proline. Protein L44 has six peptides in common with L44′ but lacks histidine. We do not known whether the proteins are the product of different genes or the results of post-translational modification of a common precursor. The existence of significant differences in their peptide maps argues in favor of the first possibility. Alternatively, a maturation process by protease cleavage of a common precursor is also possible. This mechanism has been postulated in the case of *A. salina* (van Agthoven, 1978), where two acidic proteins of different molecular weight were detected in the ribosome. However, in the case of *S. cerevisiae*, if this process is working, the sites of cleavage in the precursor have to be very close, since by SDS gel electrophoresis differences in the molecular weights of the polypeptides are not detected.

We cannot exclude other possible modification processes as responsible for the existence of three proteins. However, the most common modification mechanisms described in cellular metabolism, namely, adenylation, methylation, and acetylation, do not seem to be working in this case (F. J. Vidales and J. P. G. Ballesta, unpublished results).

In any case, whatever the mechanism responsible for the existence of the three polypeptides, the process seems to be controlled by the cellular metabolism, since the ratio of the three species can be altered by changing the metabolic conditions of the cell (growth in the presence of inhibitors) and by genetic manipulations (unpublished results and S. Zinker, personal communication).

Contrary to other eukaryotic systems, where the acidic proteins can be multiphosphorylated (Reyes et al., 1977; Tsuguri et al., 1978; Vidales et al., 1981a), the three proteins of *S. cerevisiae* are only monophosphorylated. When ribosomes are prepared carefully, only the phosphorylated forms of the proteins are detected in the particles; on the contrary, in the supernatant, they appear unphosphorylated (Zinker, 1980; Sánchez-Madrid et al., 1981b). These data suggest that phosphorylation might affect the binding process of the proteins to the particles. This idea has been tested directly by using radioactive proteins, and the results confirm that protein-deprived ribosomes bind little unphosphorylated protein under conditions in which up to 3.4 molecules of phosphorylated polypeptide per particle were bound. These results agree well with the lack of reconstituting activity of the unphosphorylated acidic proteins [see Table III and McConnell & Kaplan (1982)].

The drastic effect on the binding properties of the proteins of phosphorylation implies a pronounced change in the polypeptide conformation, also confirmed by the different susceptibility to iodination shown by the two protein forms. Under similar iodination conditions, the phosphorylated form is considerably more labeled than the unphosphorylated polypeptide.

Additional data are required to understand the metabolic significance of acidic ribosomal protein phosphorylation. A modulating mechanism of ribosome function based on a phosphorylation–dephosphorylation process of acidic proteins responding to subtle metabolic changes is unlikely. However, the yes or no response of the process on the ribosome function,

due to release of acidic proteins, is compatible with a mechanism working in extreme metabolic conditions, where partial inactivation of the ribosomes must be required. In fact, our preliminary data seem to indicate that, upon inhibition of cell growth by fluoride ions, the content of acidic protein in the ribosome decreases, and the pool of these proteins in the supernatant increases (unpublished results).

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