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CHARACTERIZATION OF TWO ACIDIC PROTEINS OF Saccharomyces cerevisiae RIBOSOME.

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SUMMARY

In Saccharomyces cerevisiae ribosomes two proteins, L44 and L45, of strong acidic character are detected. These proteins, presumably equivalent to bacterial L7 and L12, have been purified and have given a total cross reaction when tested by double immunodiffusion. Reaction with fluorescamine has shown that the amino terminal group of the polypeptide is blocked in protein L44 and free in protein L45. Tryptic analysis of the two proteins shows that three out of nine peptides are in identical position in both patterns, three more are easily related and the last three are clearly different. The data indicate that proteins L44 and L45 are closely related but not totally identical.

INTRODUCTION

The existance of highly acidic proteins in the large subunit of the ribosomes from all organisms which have been studied is well established (1-17). In eukaryotic particles several proteins of this type have been reported although their mutual relationship has not been clearly established yet. In Artemia salina and rat liver two proteins have been identify that seem to be partially related (12,13)

In Saccharomyces cerevisiae the presence of two proteins of mobility similar to bacterial L7/L12 was initially detected by standard two dimensional electrophoresis (28,15). More recently Itoh et al (18) reported the presence in yeast ribosomes of four proteins of acidic characteristics resolved by a different electrophoresis system.

In our laboratory we have shown by electrofocusing that up to eight bands can be detected in the ribosome of S.cerevisiae which upon treatment with phosphatase are reduced to three with isoelectric points around pH 4.0 (19,20)

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One of them, which we have named protein Ax, is probably not a real ribosomal protein although, when phosphorylated, it binds to the ribosome as strongly as the other acidic proteins (20,21). The other two polypeptides, L44 and L45 in the nomenclature of Kruiswijk and Planta (15) correspond to typical acidic ribosomal proteins of the L7/L12 class.

It seems, therefore, clear that in S.cerevisiae ribosomes there are two acidic proteins that can be partially phosphorylated, yielding the four spots detected by other authors (18). The characteristics of these two proteins are very similar (19,20) and since the amino acid sequence of only one of them has been reported (22), we have studied the extent of the similarity between the two of them using different chemical and immunological techniques.

MATERIALS AND METHODS

Ribosomes were obtained from Saccharomyces cerevisiae Y166 as previously described (23). Acidic proteins were extracted from the particles by washing with 1.0 M NH_4Cl in the presence of 50% ethanol (23). The extracted $SP_{1.0}$ proteins were treated with alkaline phosphatase in $(NH_4)_2 CO_3$ 10 mM pH 9.0 at 37°C for 1 h and then lyophilized. The sample was disolved in 10 mM ammoniun acetate 2 mM β-mercapto ethanol and 6 M urea, applied to a CM-cellulose and eluted with the same buffer. The CM-cellulose unretained fraction was applíd to a DEAE-cellulose column (2.5 x 16 cm) and eluted by a gradient of ammoniun acetate (10 mM to 1 M). The fractions were dialyzed against 0.05% ammonia and lyophilized. The separation of proteins L44 and Ax was carried out by filtration in Sephadex G-100 using 20 mM $NaHCO_3$ pH 8.5 and 0.5 M NaCl.

Sera against the acidic proteins were obtained from rabbits after an intramuscular injection of 200-300 µg of either purified acidic proteins or total extracted fraction $SP_{0.4}$ in complete Freund adjuvant, followed by several subcutaneous booster injections in incomplete adjuvant every 15 days. The purified protein anti-sera was tested against acidic proteins and the total protein from the $P_{0.5}$ core particles, and shown to be specific against the acidic protein. Immunodiffusión was carried out on 1% agar plates in 10 mM Tris-HCl 150 mM NaCl pH 7.4.

The estimation of N-terminal amino groups was carried out as described (24). Tryptic peptide maps were made by digesting the protein sample (200 µg) with trypsin (5 µg) in 0.2 M NH_4HCO_3 pH 8.5 for 4 h at 37°C. 100 µg of the hydrolyzed sample were applied to a Merck cellulose F_{254} plate and resolved in the first direction by electrophoresis in 4% pyridine and 1.25% acetic acid pH 5.4 at 400 v for 2h 15 min. and in the second direction by ascending chromatography in pyridine-acetic acid-1-butanol-H_2O (10/3/15/12 v/v). The dried plate was stained with 1% ninhydrine in acetone.

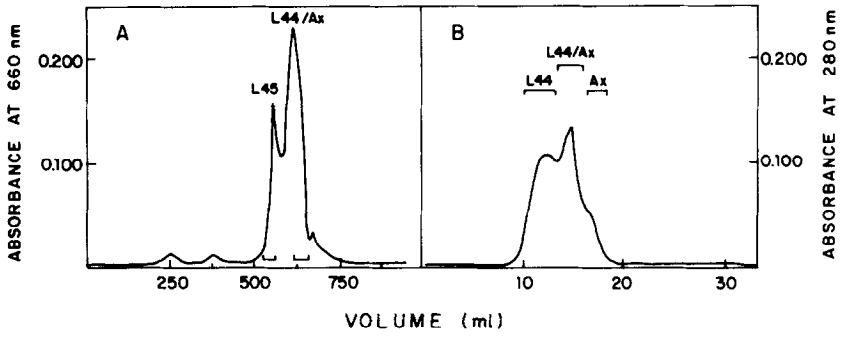

Figure 1. Separation of acidic proteins associated with S.cerevisiae ribosomes.- A) $SP_{1.0}$ proteins extracted from the ribosomes and treated as indicated in Methods were applied to a DEAE-cellulose column and eluted with a ammoniun acetate gradient (10 mM to 1 M). The proteins in the fractions collected were identified by electrofocusing. B) The fractions from the DEAE-cellulose column containing proteins L44 and Ax were pooled and applied to a Sephadex G-100 column and eluted with 20 mM CO_3HNa,NaCl 0.5 M pH 8.5.

RESULTS

Purification of proteins L44 and L45.- The three acidic proteins associated with the ribosomes of S.cerevisiae, proteins L44, L45 and Ax (19-21), show very similar physicochemical characteristics and the resolution of a mixture of the three species, which in addition can be present in different phosphorylation states (20), is not easily achieved by standard methods of column chromatography. Previously we had been able to purify the most phosphorylated from of protein Ax starting from the ethanol-ammoniun extract of the ribosomes in conditions that did not allow the resolution of protein L44 and L45 (19). Under similar conditions, but first treating the extracted proteins with alkaline phosphatase in order to reduce the number of species of polypeptides present in the sample, we have been able to separate a fraction of protein L45 by DEAE-cellulose (Fig 1A).

In order to separate protein L44 from Ax, we took advantage of the capacity of self-association shown by the acidic proteins from the ribosomes of other cells (12,25) hoping that, at low salt concentrations, protein

Ax would not associate with protein L44 which would be present as dimers or higher aggregates.

In fact, our initial attempts to separate L44 from Ax by filtration through Sephadex G-100at low salt concentration showed that all the material applied to the column came out in the excluded volume, clearly indicating that in those conditions the two proteins were able to interact forming aggregates of high molecular weight.

However, by increasing the salt concentration in the protein solution up to 0.5 M, we were able to resolve several fractions (Fig 1B). This allowed us to obtained a purified preparation of L44 and at the same time indicated that the complex formed by protein Ax and L44 is less stable than the dimers of protein L44.

Immunological relationship of protein L44 and L45.- Using antisera rised against the $SP_{0.4}$ fraction of yeast ribosomes, it was shown that protein Ax is immunologically unrelated to proteins L44 and L45, which ussually give a double precipitine band (19). When tested independently, either using the fractions from the column described above, or proteins extracted from isoelectrofocusing gels, the two proteins L44/45 give an apparently total cross reaction (Fig 2) indicating that the determinants the two proteins display when injected into rabbits are identical. We must say, however, that on one ocasion a weak spur was detected in the junction of the two precipitation lines, suggesting a possible partial crossreaction. Nevertheless, it was not possible to reproduce the effect even in conditions unfavourable to the formation of protein associations which could cover the possible structural differences between the two types of proteins, namely in the presence of 0.5% triton X-100 and 0.5 M NaCl. In all cases however it was possible to detect several consecutive bands of precipitation (Fig 2), which we have interpreted as being due to the aggregates of the proteins.

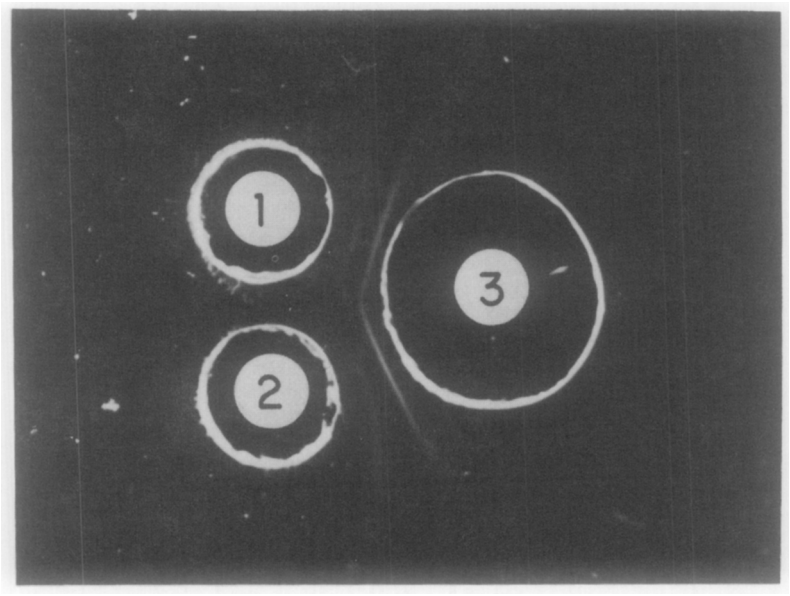

Figure 2. Immunological cross reaction of proteins L44 and L45.- Protein L44 (3 µg) in well 1 and Protein L45 (3 µg) in well 2 were tested against serum rised against purified yeast acidic proteins (well 3).

Analysis of the N-terminals amino groups of proteins L44 and L45.

The apparent immunological identity of proteins L44 and L45, which also have isoelectrical points that are clearly distinguishable by electrofocusing (19,20), recalls the situation of the bacterial proteins L7 and L12 which are only differenciated in that their amino terminal group is blocked.

We have therefore studied the state of the N-terminal groups of proteins L44 and L45. A recently developed technique (24) that applies the different way the primary amino groups of proteins react with fluorescamine depending on their position in the polypeptide and the pH of the reaction has been used. At pH 5.5 only the free N-terminal amino groups are reactive while at pH 8.5 all the amino groups will react with the fluorescamine. Comparing, then, the fluorescence of the proteins treated at pH 5.5 and pH 8.5 it is posible to estimate the state of their N-terminal amino group. In Fig 3 we have compared the reactivity of proteins L44 and L45 with fluoresca-

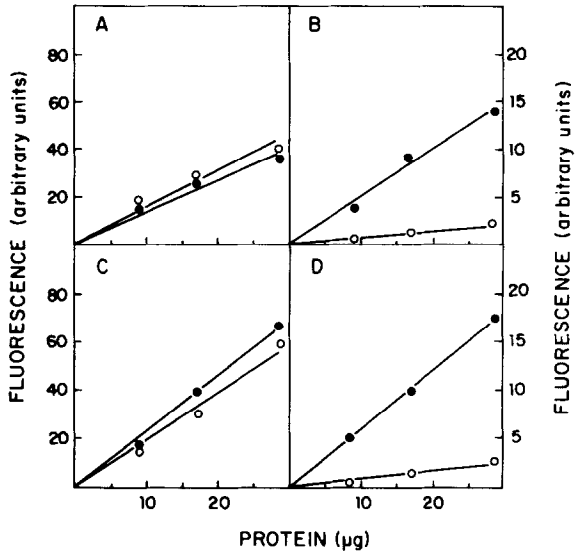

Figure 3. Reaction of proteins L44/45 from S.cerevisiae and L7/12 from E.coli with fluorescamine. A and B.- Bacterial proteins (L7 open circles, L12 filled circles) treated with fluorescamine at pH 8.5 (A) and pH 5.5 (B). C and D.- S.cerevisiae proteins (L44 open circles, L45 filled circles) treated with fluorescamine at pH 8.5 (C) and pH 5.5 (D).

mine at pH 5.5 and pH 8.5. As a positive control, proteins L7 and L12 from E.coli were also tested. It is clear from the results that L7 and L44 on one hand and L12 and L45 on the other, react in a similar way, indicating that in the first case the N-terminal amino group is blocked and in the second case it is free.

Tryptic analysis of proteins L44 and L45.- In order to confirm the identity of the proteins L44 and L45, which the immunological results seemed to indicate exist, we carried out a tryptic digestion of the proteins. The analysis of the resulting peptides showed that in fact the two proteins differ considerably in their peptide patern (Fig 4). In both cases, we detected nine peptides. Of them only three totally corresponded in the two samples, three were easily correlated as to proximity and colour after staining and three were not clearly related. Although from a tryptic analysis it is not possible to quantify the extent of similarity between the aminoacid sequence of proteins, which in fact can be closer

than the apparent disimilarity of the tryptic pattern might indicate, our data definitely show that the two proteins are not identical.

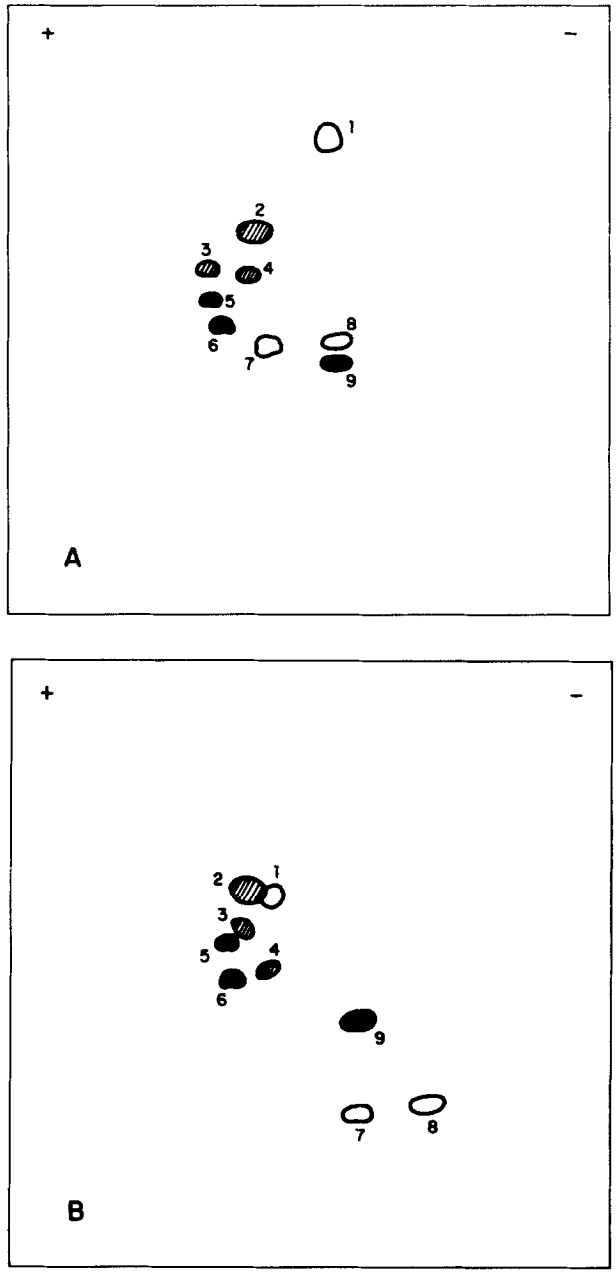

Figure 4. Tryptic analysis of proteins L44 (A) and L45 (B). Spots 5,6 and 9 are in the same position in both plates; spots 2,3 and 9 seem to be related as to their colour after staining.

DISCUSSION

Previous data have suggested a close similarity between the two acidic proteins L44 and L45 from the ribosomes of S.cerevisiae that functionally and structurally seem to be equivalent to the bacterial proteins L7 and L12 (13,20,23). Indeed, the molecular weight and amino acid composition of the two polypeptides are very similar, if not identical (13,19).

Our results using antisera rised against the acidic proteins showed a total cross-reactivity that suggested the identity of the two proteins. In A.salina, the equivalent proteins, eL12 and eL12', seem to give no more than 25% of crossreactivity when tested in a radio-immuno assay (12). The different isoelectric points of proteins L44 and L45 (19) could then be explained by the state of the amino terminal group of the two polypeptides which is blocked in L44 and free in L45 as clearly indicated by to fluorescamine test (Fig 3).

These data seemed to show a situation similar to that of the bacterial ribosome with the difference that yeast acidic proteins can be phosphorylated "in vivo" (15,19,20). The tryptic analysis, however, indicated that this was not the case, since the peptide pattern of the two proteins is clearly distinguishable. Even supposing that the differences shown by the tryptic analysis of proteins L44 and L45 exagerate the actual difference in the amino acid sequence, they are evident proof to non-identity.

The immunological cross-reactivity of proteins in the immunodiffusion test that are not identical has been shown in other instances (26) and might be interpreted by assuming similar secondary and tertiary structures of the polypeptides that display identical immunogenic determinants. In the case of lysozyme differences of up to 10 aminoacids seem to be undetected by the Ouchterlony test (27).

In spite of the differences in the amino acid sequence that the proteins L44 and L45 might have, all the data available seem to indicate

however that they play an identical functional role, as seems to be the case of bacterial L7 and L12, although definitive proof is still lacking.

From a genetic point of view, the non-identity of proteins L44 and L45 implies the existence of two genes for the acidic proteins that probably arose by duplication of the original one present in more primitive cells. The existence of two genes, on the other hand, suggests that the mechanisms regulating the number of copies of each protein present in the ribosomes depending on growth conditions (19), must differ from those that regulate the L7/L12 ratio in bacterial cells.

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