Vol. 91, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 28, 1979 Pages 643-650

AN ACIDIC PROTEIN ASSOCIATED TO RIBOSOMES OF *Saccharomyces cerevisiae* . CHANGES DURING CELL CYCLE

F. Sanchez-Madrid and J.P.G.Ballesta

Instituto de Biochimica de Macromoleculas,Centro de Biologia Molecular,
C.S.I.C. ,Canto Blanco,Madrid-34,Spain

Received August 10,1979

SUMMARY

A large proportion of protein Ax, one of the three acidic proteins detected in S.cerevisiae is found in the cell cytoplasm. The fraction of Ax associated with the ribosomes is partially released by treatment of these particles in conditions that remove the bound initiation factors but a residual amount remains tightly bound to the ribosomes.
Protein Ax is associated with the large ribosomal subunit and the amount of protein in the particles duplicates as the celle enters the stationary phase.
The amino acid composition of protein Ax is similar to those of other acidic proteins from the ribosomes of yeast and other species being very rich in alanine (21.3%),glycine (12.2%),aspartic acid (14.7%) and glumatic acid (14.3%).

INTRODUCTION

We have reported (1) the existance in ribosomes of *Saccharomyces cerevisae* of several phosphorylated acidic proteins with electrophoretic mobilities similar to those of proteins that in other prokaryotic and eukaryotic cells have been implicated in the interaction of the elongation factors with ribosomes (2-7).Two of these proteins from yeast have been called L44/45 (8), L35/36 (9) or A1 and A2 (mentioned in ref. 10) by other authors and they are involved, at least partially, in the interaction of factor EF-2 with the ribosome (11). The third protein, which we have named Ax, was not mentioned in previous reports. It is more phosphorylated than as well as immunogenically different from the other two proteins (1) and is not related to elongation factor activity (unpublished results)

The characteristics of protein Ax make it highly interesting, inducing us to explore further its relation with the ribosome. The results presented in this report seem to indicate that Ax is not a typical ribosomal protein but somewhat resembles a supernatant factor although in some conditions it is firmly bound to the ribosome.

0006-291X/79/220643-08$01.00/0

MATERIALS AND METHODS

Preparation of ribosomes.

Cells of Saccharomyces cerevisiae Y166 grown in YEPD medium (11) in a Lab-Line/S.M.S. Hi-density fermentor were broken by sea sand grinding in Buffer 1 (80 mM KCl, 12.5 mM $MgCl_2$, 1 mM DTT and 100 mM Tris-HCl, pH 7.4), the suspension was centrifuged at low speed (5.000 rpm for 20 min.) in order to remove sand and the larger cell debris and the supernatant was centrifuged again at 30.000 x g to obtain an S-30 fraction and a membrane-enriched pellet (P-30). The ribosomes were pelleted from the S-30 supernatant at 150.000 x g, resuspended in Buffer 2(500 mM NH_4Acetate, 100 mM $MgCl_2$, 5 mM B-mercaptoethanol, 20 mM Tris-HCl pH 7.4) and finally washed by centrifugation through two layers of 20% and 40% sucrose in buffer 2. The particles were kept at -80°C in buffer 1,

Ribosomes attached to membrane fragments were recovered by resuspending the P-30 fraction in buffer 1 containing 1% sodium deoxicholate (DOC). After 30 min. continuous shaking at 0°C, the sample was centrifuged for 15 min. at 30.000 x g to remove the precipitated material, which was discarded, the supernantant was centrifuged at 150.000 x g and the pelleted particles were washed as described above and resuspended in buffer 2 and kept at -80°C. Ribosomal subunits were prepared by dissociating the particles by dialysis against Buffer 3 (500 mM KCl, 5 mM $MgCl_2$, 10 mM B-mercaptoethanol, 50 mM Tris-HCl, pH 7.4) for 12 h.The particles were separated by centrifugation through a 7-35% sucrose gradient in buffer 3 at 17°C in a zonal rotor. After 5 1/2 h. at 32.000 rpm, 12 ml fractions were collected over 1M $MgCl_2$ to give a final concentration of 50 mM and those containing the subunits were pooled and centrifuged at 150.000 x g. The particles were kept in buffer 1 at -80°C.

The A_{260nm} of a solution containing 1 mg/ml is taken to be 14.00 and the molecular weight of the 80s, 60s and 40s particles to be 4.0×10^6, 2.7×10^6 and 1.3×10^6 respectively. (12).

High salt treatment of ribosomes.

In order to release the acidic proteins the ribosomes were treated in several ways: a) Ammonium-ethanol washing. The particles were treated with 0.5 M NH_4Cl in the presence of 50% ethanol as previously reported (11).
b) Removal of initiation factors. A suspension of ribosomes in buffer 2 (20-30 mg/ml) was adjusted to 0.5 M KCl, held at 0°C for 30 minutes and then centrifuged at 150.000 g for 3 hours. The supernatant containing the crude initiation factor preparation was fractionated by ammonium sulphate precipitation. Material which precipitated between 30% and 70% ammonium sulphate saturation was used as the initiation factor preparation (13).

Estimation of Ax.

The amount of protein Ax present in the different samples tested was estimated by radial immunodiffusion (14). 0.3 ml of antiserum to Ax was mixed with 5 ml of 1% agar in 10 mM Tris-HCl pH 7.4 and 150 mM NaCl at 50°C and spread on glass plates and increasing concentration of Ax were placed in 5 mm diameter holes punched in the agar layer. After developing for 18h at room temperature the plates were photographed. Measurements were made on enlarged prints; the diameter of the preciptin ring is a linear function of the logarithm of the antigen concentration.

Amino acid composition of protein Ax.

0.1 ml of protein Ax was hydrolyzed in 6N HCl for 24h at 110°C. The amino acid composition was determined in a Durrum D-500 analyzer. The

determinations were performed by Dr. M.Ugarte at the Biochemistry Department, Universidad Autonoma,Madrid.

RESULTS

Distribution of protein Ax after Ribosome dissociation. 80s ribosomes were dissociated and separated by zonal centrifugation. Total proteins, were extracted from similar amounts of ribosomes and subunits by treatment with 67% acetic acid and the amount of protein Ax present in the samples was estimated by radial immumodiffusion. Table 1 shows that the large subunit retains about 50% of the amount of Ax originally present in the 80s ribosome while the 40s particle is almost totally devoid of it. The amount of Ax present in the top fraction is lower than expected. Probably part of the protein is lost during manipulation suggesting that Ax is a "sticky" protein.

Presence of Ax in the initiation factors preparation. Initiation factors bind strongly to ribosomes from which they can be removed by washing with high salt concentrations. After ammonium sulfate fractionation proteins removed by high salt washing are used as crude intitiation factor preparations (13). Protein Ax is 50% released from the ribosomes in conditions used to prepare initiation factors and moreover, is associated only with the ammonium sulphate fraction having initiating activity(Table 2).

TABLE 1. Distribution of protein Ax after ribosome dissociation.

Fraction	µg Ax/mg particles	mol Ax/mol particle
80s	3.14	0.96
60s	2.04	0.47
40s	0.41	0.04
Gradient top	0.26[a]	--

(a) The volumen of the top fraction used corresponded to 1 mg of dissociated 80s particles.

TABLE 2. Presence of protein Ax in the crude initiation factor preparation.

Fraction	μg Ax/mg ribos.	mol Ax/mol ribos.
80s control	3.9	1.2
0.5M KCl washed 80s	2.15	0.66
Supernatant (0-30% NH_4SO_4)	0	0
Supernatant (30%-70% NH_4SO_4)	0.75	0.23

Lines 3 and 4 correspond to high salt wash proteins derived from 1mg of ribosomes fractionated by ammonium sulphate precipitation.

Presence of protein Ax in the cytoplasm. The previous data indicated that protein Ax is relatively easy to remove from the ribosomes, suggesting the possibility of an exchange at some step during protein synthesis with a pool of free protein in the cytoplasm. When the amount of protein Ax in the cytoplasm of cells collected at late exponential phase was estimated, roughly twice as much was detected in the supernatant as in the ribosomes (7.2 and 3.0 μg of Ax/mg of ribosomes respectively).

The amount of protein Ax bound to the ribosomes doubles as the cells enter stationary phase although the total amount of Ax in the cytoplasm does not change with the cell cycle (Table 3). Since the probability of an increase in the synthesis of a protein in stationary cells is low, the above results indicate loss of part of the protein Ax present in exponentially growing cells. Most cell membrane material is discarded during ribosome preparation and we therefore tested for the presence of protein Ax in this cell fraction. The material pelleted at 30.000 xg from the cell lysate was extracted with deoxycholate (DOC) and from the extracted material ribosomes were recovered. As indicated in Table 4, while the mount of protein Ax in ribosomes obtained from either

TABLE 3. Distribution of protein Ax throughout the cell cycle.

Optical Density of the culture (A_{550})	Protein Ax in:		
	Ribosomes		Supernatant
	μg Ax/mg ribos.	mol Ax/mol ribos.	μg Ax/mg ribos.
5	3.1	0.95	5.8
10	3.05	0.93	6.7
15	4.09	1.25	5.64
16	6.9	2.12	6.2

the cytoplasm or the membrane is similar, the supernatant form the membrane fraction is about 50% richer in Ax than the standard supernatant.

Chemical composition of protein Ax. The results so far presented clearly indicate that protein Ax does not behave like a typical ribosomal protein. Neverthless in some physicochemical respects it is similar to the typical ribosomal acidic proteins L44/45 (proteins A1 and A2 of other authors (see reference 10). The chemical composition of A1 and A2 has been reported previously (10) and the aminoacid analysis of protein Ax detailed in Table 5 indicates a close similarity

TABLE 4. Association of protein Ax with membrane fractions.

Sample	Protein Ax μg Ax/mg ribosom.
Membrane supernatant	11,8
Cytoplasmic supernatant	7.3
Membrane ribosomes	3,5
Cytoplasmic ribosomes	3,1

Table 5. Amino acid composition of protein Ax (moles percent)

Asp	14.7
Thr	3.2
Ser	5.45
Glu	14.3
Glr	0.15
Pro	2.1
Gly	12.2
Ala	21.3
Val	7.2
Ile	2.6
Leu	7.5
Tyr	0.5
Phe	2.6
His	Undetected
Lys	5.3
Arg	Undetected

Amino acid determinations were carried out in triplicate samples. Tryptophan and cysteine were not determined.

between the three proteins. Protein Ax has a slightly higher content of acidic aminoacids and lower content of basic aminoacids than proteins A1 and A2 in agreement with its higher acidic pK (1).

DISCUSSION

Protein Ax is a highly phosphorylated protein that appears associated to the ribosomes as well as free in the cytoplasm of Saccharomyces cerevisiae cells. In its electrophoretic mobility and chemical composition it is similar to proteins L44/45, the yeast ribosomal proteins equivalent to the bacterial proteins L7 and L12 (1,10).

In other respects however, i.e. functional role and immunogenic determinants (1), there is no the similarity between these two types of proteins.

Protein Ax is partly released from ribosomes in association with the initiation factors but 50% of it remains bound to the 60s subunits, being released in conditions that also totally remove proteins L44/45 (11). Preliminary results seem to indicate that the easy removal of Ax from the ribosomes is related to its degree of phosphorilation.

A substantial fraction of protein Ax is associated with membrane fractions, probably membrane-bound polysomes, in exponentialy growing cells. These ribosomes have the same amount of bound Ax as cytoplasmic particles but a considerably larger amount of protein easily removed simply by membrane disruption with detergent. Although an unspecific binding of protein Ax to membrane and/or to ribosomes can not be excluded, the results suggest that ribosomes involved in protein synthesis might be enriched in an easily removable fraction of protein Ax. Moreover, the amount of Ax more tightly bound to ribosomes duplicates as the cells enter the stationary phase. These date are compatible with a regulatory role of protein Ax. However, no direct effect of this protein on "in vitro" ribosomal functions has so far been detected (10).

Taken together, the present results suggest that protein Ax is not a typical ribosomal protein but rather that it behaves like a supernatant factor which, in some conditions, can be tightly bound to the large ribosomal subunit. The chemical composition of protein Ax makes it even more interesting. Its resemblance to the acidic proteins present in ribosomes (10) as well as in contractile systems (15) is noteworthy and might be the clue to its role in ribosome function. Nevertheless, the close chemical similarity of most acidic proteins so far analyzed raises the question of whether those chemical characteristics are structural requirements related not to the function but to the physicochemical stability of the molecule. In any case, the recent report (16) describind an "in vitro" system prepared from yeast that translates natural exogenous mRNA and the availability of antisera to protein Ax provide us with tools to explore the functional significance of this protein in the protein synthesis machinery. The results of these experiments will probably clarify the relationship of protein Ax with the ribosome.

ACKNOWLEDMENTS

This work was supported by an Institutional grant to the Centro de Biología Molecular from Comision Administradora del Descuento Complementario (Instituto Nacional de Previsión) and personal grants from Comision Asesora de Investigación Cientifica y Técnica. We thank Dr. D. Vázquez for reading and commenting the manuscript and Dra. M. Ugarte for the analysis of amino acids.

REFERENCES

1. Sanchez-Madrid, F., Conde, P., Vázquez, D. and Ballesta, J.P.G. (1979) Biochem. Biophys. Res. Commun. 87, 281-291.
2. Möller, W., Groene, A., Terhorst, C. and Amons, R. (1972) Eur. J. Biochem. 25, 5-12.
3. Stöffler, G., Wool, I.G., Lin, A. and Rak. K.H. (1974) Proc. Nat. Acad.Sci. 71, 4723-4726.
4. Möller, W., Slobin, L.I., Amons, R. and Richter, D. (1975) Proc, Nat. Acad. Sci. 72, 4744-4748.
5. Horak, I. and Schiffmann, D. (1977) Eur. J. Biochem. 79, 375-380.
6. Reyes, R., Vázquez, D. and Ballesta, J.P.G. (1977) Eur. J. Biochem. 73, 25-31.
7. Leader, D.P., Colia, A.A. and Falmy, L.H. (1978) Biochem. Biophys. Res. Commun, 83, 50-58.
8. Kruiswijk,I. and Planta, R.J. (1975) FEBS Lett. 58, 102-105.
9. Zinker, S. and Warner, J.R. (1976) J. Biol. Chem. 251, 1799-1807.
10. Tsurugi, K., Collatz, E.,Todokoro, K.,Ulbrich, N., Lightfoot, H.N. and Woll, I.G. (1978) J. Biol. Chem. 253, 946-955.
11. Sanchez-Madrid, F., Conde, P. and Ballesta, J.P.G. (1979) Eur. J. Biochem. 98, 409-416
12. Petterman, M.L. (1964). In the Physical and Chemical propeties of ribosomes, pp. 121-133. Elsevier, Amsterdam.
13. Fresno, M. Carrasco, L. and Vázquez, D. (1976) Eur. J. Biochem. 68, 355-364.
14. Mancini, G., Carbonara, A.O. and Heremans, J.F. (1965) Immunochemistry 2, 235-254.
15. Amons, R., Van Agthoven,A. Pluijms, W. and Möller, W. (1978), FEBS Lett. 86, 282-284.
16. Gasior, E., Herrera, F., Sadnik, I., MacLaughlin, C.S. and Moldave, K. (1979) J. Biol. Chem. 254, 3965- 3976.