Modification of Yeast Ribosomal Proteins

PHOSPHORYLATION

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Two-dimensional polyacrylamide-gel electrophoretic analysis of yeast ribosomal proteins labelled *in vivo* with $^{32}\text{PO}_4^{3-}$ revealed that the proteins S2 and S10 of the 40S ribosomal subunit, and the proteins L9, L30, L44 and L45 of the 60S ribosomal subunit, are phosphorylated *in vivo*. Most of the phosphate groups appeared to be linked to serine residues. The number of phosphate groups per molecule of phosphorylated protein species ranged from 0.01 to 0.79. Since most of the phosphorylated ribosomal proteins appear to associate with the pre-ribosomal particles at a very late stage of ribosome assembly, phosphorylation is more likely to play a role in the functioning of the ribosome than in its assembly.

Ribosomal proteins appear to be modified *in vivo* by the attachment of methyl, acetyl and/or phosphate groups. Whereas methylation and acetylation of ribosomal proteins take place both in bacteria (Comb *et al.*, 1966; Terhorst *et al.*, 1972*a,b*; Chang *et al.*, 1974; Alix & Hayes, 1974; Chang & Chang, 1974, 1975) and in higher cells (Chang *et al.*, 1976; Vandrey *et al.*, 1976; Cannon *et al.*, 1977), phosphorylation seems to be specific for eukaryotic ribosomes (Gordon, 1971; Krystosek *et al.*, 1974; Gressner & Wool, 1974*a,b*; Barela & Kizer, 1974; Olson *et al.*, 1974; Rankine & Leader, 1975; Zinker & Warner, 1976; Becker-Ursic & Davies, 1976; Traugh & Porter, 1976; Rankine *et al.*, 1977; Hébert *et al.*, 1977).

The biological significance of the modification of ribosomal proteins is still an open question. However, two possibilities are obvious: these modifications may be required for the proper functioning of the ribosome in protein biosynthesis and/or may play a role in ribosome assembly. Studies on the course of assembly of yeast ribosomal subunits by labelling kinetics have revealed that the various ribosomal proteins can be divided into two sets: one set associates with precursor rRNA at a relatively early stage of ribosomal maturation; the second set assembles with the immature ribosome relatively late during ribosome maturation (Kruiswijk *et al.*, 1978*a*). To reveal a possible function of modification of yeast ribosomal proteins in ribosome assembly we have investigated the type and degree of modification of the various proteins of the two sets.

In the present paper we present data on the phosphorylation of these proteins. The methylation of the ribosomal proteins is described in the next paper (Kruiswijk *et al.*, 1978*c*).

Materials and Methods

Cell culturing and labelling conditions

Saccharomyces carlsbergensis, strain S74, was grown in a medium containing 10 g of glucose, 1 g of bacteriological peptone, 3 g of malt extract and 2 g of yeast extract per litre, at 29°C overnight in the presence of inorganic $^{32}\text{PO}_4^{3-}$ (The Radiochemical Centre, Amersham, Bucks., U.K.; 135 Ci/mg of phosphate; 0.13 mCi/ml). Cells were harvested by centrifugation at 10000g for 15 min when the culture had reached the exponential phase (1×10^7 – 2×10^7 cells/ml).

Isolation of ribosomal subunits

Homogenization of labelled yeast cells by shaking with glass beads and the subsequent removal of cell debris from the homogenate was performed as described previously (Kruiswijk & Planta, 1974). Ribosomes were isolated and separated into subunits by sucrose-gradient centrifugation as described by Kruiswijk *et al.* (1978*a*). The 40S and 60S ribosomal subunits were pelleted from the sucrose solution by centrifugation at 196000g for 20 h at 4°C in the Spinco SW-41 rotor.

Extraction and polyacrylamide-gel electrophoretic separation of ribosomal proteins

Protein extraction was performed with 2-chloroethanol as described by Fogel & Sypherd (1968; see also Kruiswijk & Planta, 1974). Two-dimensional gel electrophoresis of ribosomal proteins was performed as described by Kruiswijk & Planta (1974).

One-dimensional polyacrylamide-gel electrophoresis was performed on disc gels of 18% (w/v) acrylamide (17 cm × 0.5 cm) at pH 4.6. Gel composition and electrophoresis buffer were the same as used in the second dimension of the two-dimensional electrophoresis system (Kruiswijk & Planta, 1974). A spacer gel of 2.5% (w/v) acrylamide (2 cm × 0.5 cm) at pH 6.9 was used. Electrophoresis was carried out at 0.7 mA/gel for 40 h at 4°C.

Slab gels as well as disc gels were stained with Coomassie Brilliant Blue as described by Kruiswijk & Planta (1974, 1975).

Identification of phosphorylated amino acid residues

Freeze-dried ribosomal proteins were dissolved in 6 M-HCl and hydrolysed in a N₂ atmosphere at 105°C in sealed ampoules for 3 h. The hydrolysate was dried *in vacuo* and dissolved in 2.5% (v/v) formic acid/7.8% acetic acid, pH 1.85 (Barden & Labrie, 1973). The solution was applied to Whatmann 3MM paper and subjected to electrophoresis at 5000 V for 80 min in 2.5% (v/v) formic acid/7.8% acetic acid (pH 1.85) in a Gilson high-voltage Electrophorator. [³²P]P_i, phosphoserine and phosphothreonine were used as markers. After electrophoresis the paper was dried at 40°C and stained with ninhydrin/cuprinite reagent (Moffat & Lyttle, 1959).

Purification of rRNA

RNA was extracted from ³²P-labelled ribosomes with phenol/sodium dodecyl sulphate as described by Retel & Planta (1967). The major rRNA species were separated by centrifugation on 5–20% (w/v) sucrose gradients in 0.1 M-NaCl/0.01 M-disodium EDTA (pH 5.0). Centrifugation was performed during 16 h at 24000 rev./min in a SW27 rotor (Beckman). The relevant fractions were pooled and precipitated with ethanol. The precipitates were collected by centrifugation at 3000g for 15 min, dissolved in water and used for the determination of the specific radioactivity of phosphate.

Radioautography and assay for radioactivity

Radioautography of dried slab gels and paper electrophoretograms was performed on Sakura X-ray medical films for 1–14 days.

Samples were counted for radioactivity in a toluene-based scintillation fluid containing 3% (v/v) NCS (Nuclear-Chicago Solubilizer, supplied by Amersham/Searle Corp.), 12.5 g of 2,5-diphenyl-oxazole and 125 mg of 1,4-bis-(5-phenyloxazol-2-yl)-benzene per litre. Radioactivity was measured in a mark II liquid scintillation counter (Nuclear-Chicago).

Results

Detection of phosphorylated ribosomal proteins

Radioautographs of polyacrylamide gels of ³²P-labelled proteins extracted from the 40S and the 60S subunits of yeast are shown in Figs. 1 and 2 respectively. As can be seen both subunits do contain phosphorylated protein species. The identity of these modified proteins can be deduced by comparing the radioautographs with the corresponding tracings of the stained gels also shown in Figs. 1 and 2. The radioactive spots do not coincide precisely with the stained spots. This is due to a change in electrophoretic mobility, caused by the phosphorylation of only a portion of each particular protein (Gressner & Wool, 1974a; Rankine & Leader, 1975; Zinker & Warner, 1976). Nevertheless, we can conclude that in the small subunit the proteins S2 and S10 and in the large subunit the proteins L9, L30 and L44/L45 are phosphorylated. On the radioautograph of the 40S-subunit proteins at least two spots are present in the region where protein S10 is located (Fig. 1). The most likely explanation for this phenomenon is the existence of multiple phosphorylated derivatives of this protein, which differ in the number of phosphate groups per protein molecule.

The phosphorylated proteins found in purified yeast ribosomal subunits appear to be the only ones present in yeast ribosomes. Analysis of the proteins isolated from undissociated 80S ribosomes did not reveal any additional phosphorylated species (results not shown).

Identity of the phosphorylated amino acid residues

To determine whether, as in higher eukaryotes, the phosphate groups present in yeast ribosomal protein are mainly bound to serine and threonine residues, an acid hydrolysate of the proteins of both the 40S and the 60S subunits was analysed by high-voltage paper electrophoresis (Fig. 3a). If we assume an equal loss of phosphoserine and phosphothreonine during hydrolysis of the protein, radioactivity measurements indicate that more than 90% of the original phosphate groups are bound to serine. A very low amount of phosphothreonine is present. The additional spots visible on the radioautograph (spots A and B in Fig. 3a) represent P_i and acid-hydrolysis products of RNA, contaminating the ribosomal protein preparations (Fig. 3b). Part of the P_i also arises from hydrolysis of phosphoester bonds in the ribosomal proteins (Bylund & Huang, 1976).

Degree of phosphorylation of yeast ribosomal proteins

Since it is impossible to assess the absolute degree of phosphorylation of individual ribosomal proteins after two-dimensional separation, we determined the

Fig. 1. *Phosphorylated proteins of the 40S ribosomal subunits*

Ribosomal proteins were extracted from 40S ribosomal subunits uniformly labelled with [^{32}P]P_i. The ribosomal proteins were separated by two-dimensional polyacrylamide-gel electrophoresis (Kruiswijk & Planta, 1974, 1975). The stained slab gels were exposed to an X-ray medical film for 7 days. (a) Radioautograph of ^{32}P -labelled 40S-subunit proteins. The arrows indicate the positions of the related spots on the stained gel. (b) Tracing of the stained ribosomal protein pattern on the slab gel.

Fig. 2. *Phosphorylated proteins of the 60S ribosomal subunit*

For experimental data see the legend to Fig. 1. The second run was carried out after two separate first-dimensional runs with samples of the same protein preparation. The anodic part of the first-dimensional gel, electrophoresed at a decreased voltage (Kruiswijk & Planta, 1975), was applied on the slab gel together with the cathodic part of the first-dimensional gel electrophoresed at standard voltage (Kruiswijk & Planta, 1974). This was necessary because of the aberrant electrophoretic behaviour of the strongly acidic proteins L44 and L45 (Kruiswijk & Planta, 1975). (a) Radioautograph of ^{32}P -labelled 60S-subunit proteins. Arrows indicate the position of the related stained spots. (b) Tracings of the stained two-dimensional protein pattern.

Fig. 3. High-voltage paper electrophoresis of an acid hydrolysate of ^{32}P -labelled ribosomal proteins (a) and of rRNA (b)

Ribosomal proteins were hydrolysed and subsequently subjected to high-voltage paper electrophoresis as described in the Materials and Methods section. rRNA, prepared from ^{32}P -labelled ribosomes by phenol/sodium dodecyl sulphate extraction, was treated with HCl and the hydrolysate was electrophoresed in parallel with the protein hydrolysate. ^{32}P as well as non-labelled phosphoserine and phosphothreonine were used as markers. The positions of the marker amino acids were made visible by spraying the electrophoretogram with ninhydrin. ^{32}P -labelled compounds were made visible by radioautography. For details of spots A and B, see the text. The identity of the faint spots detectable on the radioautograph between A and phosphothreonine could not be established.

degree of phosphorylation after one-dimensional gel-electrophoretic separation of either 40S- or 60S-subunit proteins. The densitometric scans of the stained disc gels are shown in Fig. 4. Because the electrophoretic conditions were identical with those used in the second dimension of a two-dimensional separation, the identity of the various bands can easily be established (Kruiswijk *et al.*, 1978b). The areas of the bands representing proteins L44+b, L45+L5, L9, L30+L29, S2 and S10+S11+S12 were determined by weighing the chart papers. The molar amount of the phosphorylated protein species in each of these bands was then calculated by using the calibration curve of

protein mass versus staining intensity (Fig. 5), and the molecular weights of the individual proteins were determined by us (Kruiswijk *et al.*, 1978b). In cases where a particular band contained more than one protein species, these were assumed to be present in equimolar amounts. The molar amount of phosphate in each protein band was calculated from the total amount of radioactivity in that band and the specific radioactivity of phosphate in uniformly ^{32}P -labelled rRNA isolated from the same cells as the protein (Table 1). The data in Table 1 show the degree of phosphorylation to range from 0.01 to 0.79 mol of phosphate/mol of phosphorylated protein species.

Discussion

The results in this paper demonstrate that in yeast the ribosomal proteins S2, S10, L9, L30, L44 and L45 are phosphorylated *in vivo*. Except for protein S2, all these ribosomal protein components associate with the immature ribosome at a relatively late stage of ribosome maturation and probably not before entry of the pre-ribosomal particles into the cytoplasm (Kruiswijk *et al.*, 1978a). On the basis of these results, we suggest that this type of modification of the yeast ribosomal proteins is more likely to play a role in the functioning of the ribosome than in its assembly. Support for this suggestion can be obtained from a comparison of the phosphorylated yeast ribosomal proteins with similar modified proteins found in other eukaryotic ribosomes. In the small ribosomal subunits of rat liver cells (Gressner & Wool, 1974a) and rabbit reticulocytes (Traugh & Porter, 1976) only one ribosomal protein (designated as S6 and S13 respectively) was phosphorylated *in vivo*, whereas the small subunits of Krebs II ascites cells appear to contain two phosphorylated proteins (S2 and S6) (Rankine *et al.*, 1977; Leader & Coia, 1977). These phosphorylated small subunit proteins of mammalian cells probably are analogous to yeast protein S10, since they have similar electrophoretic mobilities as well as very similar molecular weights (about 29000; Howard *et al.*, 1975). Phosphorylation of rat liver protein S6 appears to be strongly stimulated by cycloheximide and puromycin (Gressner & Wool, 1974b). Stimulation of the phosphorylation of protein S6 in regenerating rat liver has also been reported (Gressner & Wool, 1974a). Both observations point to a possible role of this modification of protein S6, and by analogy yeast protein S10, in biosynthesis.

The same reasoning applies to the phosphorylation of the yeast proteins L44 and L45. Similar strongly acidic proteins have been found on the large ribosomal subunits of rat liver (Sherton & Wool, 1974), the brine shrimp *Artemia salina* (Möller *et al.*, 1975) and Krebs II ascites cells (Rankine *et al.*, 1977; Leader & Coia, 1977). These strongly acidic proteins of eukaryotic

Fig. 4. One-dimensional polyacrylamide-gel electrophoretic separation of 40S and 60S ribosomal subunit proteins. ^{32}P -labelled proteins of the 40S and 60S ribosomal subunits were separated by one-dimensional gel electrophoresis as described in the Materials and Methods section. After thorough staining, the gels were scanned on the Vitatron Universal Photometer at 544 nm. Subsequently the gels were cut into 1 mm slices and counted for radioactivity. Peaks I–VI indicate the positions of ^{32}P -labelled protein bands. Densitometric (—) and radioactivity (●) profiles of the ribosomal proteins of the 60S (a) and 40S (b) subunits are shown.

ribosomes most probably are analogous to the *Escherichia coli* ribosomal proteins L7 and L12, which are involved in the guanosine triphosphatase reaction in protein biosynthesis (Möller, 1974). Finally, the observation that only a fraction of a particular ribosomal protein species is modified by phosphorylation (see Table 1) is compatible with the involvement of the phosphorylation in the process of protein biosynthesis.

When this work was in progress, Zinker & Warner (1976) and Hébert *et al.* (1977) reported on the phosphorylation of the ribosomal proteins in the related yeast *Saccharomyces cerevisiae*. Their results are only partially consistent with our observations. The acidic phosphorylated protein P3 and the basic

phosphorylated protein P2 found by Zinker & Warner (1976) are compatible with the phosphorylated species S2 and S10 respectively described in this paper. In addition, the phosphorylated protein P5 found in *Saccharomyces cerevisiae* is analogous to the strongly acidic protein couple L44/L45, which was found to be phosphorylated also in *Saccharomyces carlsbergensis*. On the other hand, Zinker & Warner (1976) did not observe analogues of the phosphorylated large subunit proteins L9 and L30 found in *Saccharomyces carlsbergensis*, whereas they detected two phosphorylated protein species (a neutral protein P1 on the 60S subunits and a minor protein component on the polyribosomes) in *Saccharomyces cerevisiae* that were not found in our studies. These

Table 1. *Degree of phosphorylation of yeast ribosomal proteins*

The amount (mol) of a particular phosphorylated protein species present in the one-dimensional disc gel (see Fig. 4) was calculated as described in the text. Since we uniformly labelled yeast cells with [^{32}P]P, during at least five generation times the mol of phosphate groups present in a particular band (I–IV in Fig. 4) could be calculated from the total amount of radioactivity in that band and the specific radioactivity of phosphate derived from analysis of rRNA isolated from the same culture. The specific radioactivity of rRNA [^{32}P radioactivity (d.p.m./mg of RNA)] was calculated from the A_{260} (1 mg of rRNA/ml has an absorbance of 25 units) and radioactivity measurements by liquid-scintillation counting (see the Materials and Methods section). Taking into account the average mol.wt. of the nucleotides (1 mg of ribonucleotides corresponds to 1/350 nmol of phosphate), the specific radioactivity of the phosphate in the rRNA can be calculated.

Protein band	Protein species	Mol.wt.	$10^{-11} \times$ Protein (mol)	$10^{-11} \times$ Phosphate (mol)	Phosphate (mol)/protein (mol)
I	L44	10700	3.17	2.50	0.79
II	L45	10700	17.35	2.70	0.16
III	L9	26000	19.85	0.35	0.02
IV	L30	22200	18.85	0.09	0.01
V	S2	28500	13.15	2.60	0.20
VI	S10	29000	13.15	5.00	0.38

Fig. 5. Relationship between amount of protein and staining intensity with Coomassie Brilliant Blue

Various amounts of proteins L17 and S26, purified by phosphocellulose column chromatography (T. Kruiswijk & R. J. Planta, unpublished results), were applied on separate one-dimensional 18% polyacrylamide disc gels and electrophoresed as described in the Materials and Methods section. After electrophoresis, the gels were thoroughly stained and scanned on the Vitatron Universal Photometer at 544 nm. The areas of the peaks on the densitograms were determined by weighing, and plotted against the amount of protein applied to the gel. ○, Protein L17; ●, protein S26.

differences may represent genuine species differences between the two yeast strains. Quite different results were obtained by Hébert *et al.* (1977) in analysing the phosphorylation of the ribosomal proteins of *Saccharomyces cerevisiae*. These authors did not observe phosphorylation of the strongly acidic proteins L44/L45 of the 60S subunits, neither did they detect a phosphorylated protein similar to the 40S-subunit protein S10 from *Saccharomyces carlsbergensis*. The rather controversial results obtained by Hébert *et al.* (1977) cannot be easily explained but

they may reflect a difference in growth phase of the yeast cells.

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