Modification of Yeast Ribosomal Proteins

METHYLATION

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Two-dimensional polyacrylamide-gel electrophoretic analysis of yeast ribosomal proteins uniformly labelled *in vivo* with [*methyl*-³H]methionine and [¹-¹⁴C]methionine revealed that four ribosomal proteins are methylated, i.e. proteins S31, S32, L15 and L41. Lysine and arginine appear to be the predominant acceptors of the methyl groups. The degree of methylation ranges from 0.09 to 0.20 methyl group per modified ribosomal protein species.

Modification of ribosomal proteins by methylation has been observed in a number of organisms, from *Escherichia coli* (Comb *et al.*, 1966; Terhorst *et al.*, 1972*a,b*; Chang *et al.*, 1974; Chang & Chang, 1974, 1975; Alix & Hayes, 1974) to higher eukaryotic systems like HeLa cells (Chang *et al.*, 1976; Vandrey *et al.*, 1976). The biological significance of this modification has not been clarified so far.

In the preceding paper (Kruiswijk *et al.*, 1978*c*) we showed that the yeast ribosomal proteins that are phosphorylated *in vivo* belong to the group of proteins that assemble late during ribosome maturation. This led us to the suggestion that phosphorylation is more likely to be of importance for the functioning of the ribosome than for ribosome assembly. In the present paper we extend our observations on the modification of yeast ribosomal proteins to methylation. Several yeast ribosomal protein species were methylated *in vivo*. All of them again are proteins assembling relatively late with the immature ribosome. Thus, like phosphorylation, the possible functional role of methylation of yeast ribosomal proteins most likely lies in the functioning rather than in the assembly of the ribosome.

Materials and Methods

Culturing and labelling conditions

Saccharomyces carlsbergensis, strain S74, was grown overnight at 29°C in a synthetic medium, as described in the preceding paper (Kruiswijk *et al.*, 1978*c*), in the presence of [*methyl*-³H]methionine (The Radiochemical Centre, Amersham, Bucks., U.K., 6 Ci/mmol; 0.8 µCi/ml) and [¹-¹⁴C]methionine (The Radiochemical Centre; 60 mCi/mmol; 0.2 µCi/ml). Purine nucleosides (1 mM) and sodium formate (0.01 M) were added to prevent purine-ring labelling (Klootwijk, 1974). Cells were harvested by centri-

fugation at 10000*g* for 15 min, when the culture had reached the exponential phase (1×10^7 – 2×10^7 cells/ml).

Isolation of ribosomal subunits

Ribosomal subunits were isolated from labelled yeast cells as described by Kruiswijk *et al.* (1978*c*). After sucrose-gradient centrifugation of the ribosomes, 40S and 60S ribosomal subunits were precipitated from the appropriate sucrose-gradient fractions with 0.7 vol. of ice-cold 96% ethanol at –20°C. The precipitates were collected by centrifugation at 3000*g* for 30 min at 4°C.

Extraction and polyacrylamide-gel-electrophoretic analysis of ribosomal proteins

Protein extraction was carried out with 2-chloroethanol as described previously (Kruiswijk & Planta, 1974). Two-dimensional polyacrylamide-gel electrophoresis of the ribosomal proteins was performed as described previously (Kruiswijk & Planta, 1975). One-dimensional polyacrylamide-gel electrophoresis was performed on 18% gels at pH 4.6 as described by Kruiswijk *et al.* (1978*c*). Gels were stained with Coomassie Brilliant Blue as described by Kruiswijk & Planta (1974).

Identification of methylated amino acid residues

Acid hydrolysis of the ribosomal proteins from 40S and 60S ribosomal subunits and subsequent high-voltage paper electrophoresis of the hydrolysates was performed as described by Kruiswijk *et al.* (1978*c*). Electrophoresis was carried out in a buffer containing 0.05 M-sodium borate (pH 9.2) at 6000 V for 90 min.

Purification of rRNA

rRNA was extracted from the [^3H]methyl-labelled ribosomes and the rRNA components were separated as described (Kruiswijk *et al.*, 1978c). Alkaline hydrolysis of rRNA was performed in 1M-KOH for 16h at 37°C (Klootwijk & Planta, 1973). The digest was neutralized and fractionated on a DEAE-cellulose column at pH 7.8 (Retèl *et al.*, 1969).

Assay for radioactivity

Gel fragments and paper strips were counted for radioactivity as described by Kruiswijk *et al.* (1978c). Radioactivity of rRNA solutions and of column eluate fractions was measured in a dioxan-based scintillation liquid (Klootwijk, 1974).

Results

Identification of methylated ribosomal proteins

Yeast cells were labelled with [$\text{methyl-}^3\text{H}$]methionine and [$1\text{-}^{14}\text{C}$]methionine during at least five generations. The ribosomal proteins were extracted from the 40S and 60S subunits and separated by two-dimensional polyacrylamide-gel electrophoresis. The protein spots were punched from the stained gel slabs (Kruiswijk & Planta, 1974, 1975; see also the schematic tracings in Figs. 1 and 2) and counted for ^3H and ^{14}C radioactivity. Since both ^3H and ^{14}C radioactivity will be incorporated into methionine residues, whereas only ^3H label can be incorporated into additional methyl groups, a relatively high $^3\text{H}/^{14}\text{C}$ ratio indicates the presence of methylated residues in the particular ribosomal protein species. Such relatively high $^3\text{H}/^{14}\text{C}$ ratios are found for the proteins S31 and S32 (Fig. 1), as well as for the proteins L15 and L41 (Fig. 2). In the case of the proteins S32 and L41 the $^3\text{H}/^{14}\text{C}$ ratio is disproportionately high, owing to a very low methionine content of these protein species.

Identity of methylated amino acid residues

To determine the identity of the methylated amino acid residues, ribosomal proteins isolated from the 40S and 60S subunits were hydrolysed in 6M-HCl and the hydrolysates analysed by high-voltage paper electrophoresis. Methylated amino acids show up as peaks of material containing only ^3H and no ^{14}C radioactivity. Fig. 3 shows that both the 40S- and the 60S-subunit proteins give rise to such peaks only at the positions where methylated arginine and methylated lysine residues migrate. It is impossible to discriminate between mono-, di- and tri-methyl-lysine, since these methylated amino acids migrate at the same position in the electrophoretic system used (Alix & Hayes, 1974). The same applies to mono- and

Fig. 1. Methylated ribosomal proteins of the 40S subunit in yeast

Ribosomal proteins of the 40S subunit were isolated from yeast cells uniformly labelled with [$\text{methyl-}^3\text{H}$]methionine and [$1\text{-}^{14}\text{C}$]methionine. The $^3\text{H}/^{14}\text{C}$ ratio of the individual protein components was determined as described in the text. A high ratio indicates the presence of methylated amino acid residues in the protein species concerned.

di-methylarginine. Apart from methionine and its oxidized derivative methionine sulfoxide, an unidentified compound (X) is present in the region of the neutral amino acids (Fig. 3). However, compound X is already present in the original sample of [$\text{methyl-}^3\text{H}$]methionine (result not shown), although the amount of compound X further increases on treatment of methionine with HCl. Most likely, therefore, compound X is a derivative of methionine.

Thus we may conclude that lysine and arginine are the predominant methylated amino acids in yeast ribosomal proteins. The methylated 40S-subunit proteins contain almost exclusively methylated lysine [cf. panel (a) of Fig. 3].

Degree of methylation of yeast ribosomal proteins

The number of methyl groups present in each modified ribosomal protein was estimated as follows.

The 40S- and 60S-subunit proteins were separated on long 18% polyacrylamide gels at pH 4.6. Before cutting the gels into 1 mm slices and counting for ^3H and ^{14}C radioactivity, the gels were stained thoroughly and scanned densitometrically (results not shown). The identity of the stained protein bands could easily be established since the composition of the gels was identical with that of the second-dimension gel of the standard two-dimensional electrophoresis system

Fig. 2. Methylated ribosomal proteins of the 60S subunit in yeast

See legend to Fig. 1 for experimental details. The $^3\text{H}/^{14}\text{C}$ ratio of the strongly acidic proteins L44 and L45 was determined after two-dimensional separation at decreased voltage (Kruiswijk & Planta, 1975) of ribosomal proteins extracted from undissociated 80S ribosomes isolated from the same culture.

(Kruiswijk & Planta, 1974; Kruiswijk *et al.*, 1978a). The molar amount of a methylated protein species in a particular band of the gel was calculated from its molecular weight (Kruiswijk *et al.*, 1978b) and the corresponding peak area on the densitogram. Subsequently the specific radioactivity of the methyl groups was determined. For this purpose 26S rRNA isolated from the same ^3H - and ^{14}C -labelled culture was hydrolysed in alkali and the hydrolysate was applied to a DEAE-cellulose column. About 14% of ^3H radioactivity is found in mononucleotides (result not shown). Since this value indicates that all ^3H radioactivity has been incorporated as methyl groups (Klootwijk & Planta, 1973), it is possible to calculate the specific radioactivity of the methyl groups as described in the legend to Table 1. Finally, from the specific radioactivity of the methyl group calculated in this way and the ^3H and ^{14}C radioactivity in the one-dimensional gel, mol of methyl group per mol of methylated protein species can be calculated (see legend to Table 1). Table 1 shows that the degree of methylation of the modified ribosomal protein species ranges from 0.09 to 0.20 mol of methyl group

Fig. 3. Identification of the methylated amino acids in yeast ribosomal proteins

Ribosomal proteins labelled with [methyl- ^3H]methionine ($\circ$) and [^{14}C]methionine ($\bullet$) were hydrolysed in 6M-HCl and the hydrolysate was subsequently analysed by high-voltage paper electrophoresis as described in the Materials and Methods section. The following amino acids were electrophoresed in parallel: methionine (Met), 1-methylhistidine (1-Me-His), 3-methylhistidine (3-Me-His), dimethylarginine (Me-Arg) and monomethyl-lysine (Me-Lys). After electrophoresis, the paper was dried and sprayed with ninhydrin to detect the position of the marker amino acids. The position of methionine sulfoxide (MSO) was deduced by comparing our results with those of Chang & Chang (1975) and Alix & Hayes (1974). The paper was cut into 0.5 cm strips and counted for radioactivity. Electrophoretograms of the hydrolysates of 40S-ribosomal-subunit proteins (a) and of the 60S-ribosomal-subunit proteins (b) are shown.

per mol of protein species, indicating a microheterogeneity of yeast ribosomes with respect to the methylation of the ribosomal proteins.

Discussion

Proteins S31 and S32 of the small ribosomal subunit, and proteins L15 and L41 of the large ribosomal subunit of yeast appear to be methylated *in vivo*. Our findings are compatible with a study on the methylation of the 60S subunit proteins in the related yeast *Saccharomyces cerevisiae* (Cannon *et al.*, 1977). The number and distribution of methylated ribosomal proteins seem to be characteristic for eukaryotic ribosomes. In HeLa cells, too, both the 40S and the

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

The molar amount of a methylated ribosomal protein species was calculated as described in the text. The specific radioactivity of the methyl groups can be calculated by using the formula: $[(^3\text{H d.p.m./mg of rRNA}) \times \text{mol.wt. of rRNA}] / A$, in which $^3\text{H d.p.m./mg of rRNA}$ is the specific radioactivity of 17S or 26S+5.8S rRNA. The molecular weight of 17S and 26S+5.8S rRNA and the number of methyl groups in the respective rRNA species (A) are published elsewhere (Brand & Planta, 1975; Klootwijk & Planta, 1973). We arrived at a mean value of 18.9×10^9 d.p.m. of ^3H /mmol of methyl group. The amount of ^3H radioactivity, representing methyl groups attached to lysine and arginine residues in a particular protein band on the gel [indicated as *methyl- ^3H* (d.p.m.) in the Table] is calculated from: $\sum ^3\text{H d.p.m.} - \sum ^{14}\text{Cd.p.m.} \times ^3\text{H}/^{14}\text{C}$, in which the average $^3\text{H}/^{14}\text{C}$ ratio is calculated from the $^3\text{H}/^{14}\text{C}$ ratios of non-methylated ribosomal proteins. From the *methyl- ^3H* radioactivity in a particular band and the specific radioactivity of the methyl groups, the molar amount of methyl groups can be calculated. Combining this value with the molar amount of the methylated protein species gives the degree of methylation of that particular ribosomal protein.

Protein species	$10^{-11} \times \text{Mol of protein}$	<i>methyl-^3H</i> (d.p.m.)	$10^{-11} \times \text{Mol of methyl group}$	Mol of methyl group/mol of protein
S31	30.17	735	3.89	0.13
S32	13.69	310	1.64	0.12
L15	23.65	876	4.64	0.20
L41	23.76	407	2.15	0.09

60S ribosomal subunit each contain two methylated protein species (Chang *et al.*, 1976; Vandrey *et al.*, 1976). One of the methylated 60S-subunit proteins of this organism resembles the methylated yeast ribosomal protein L15 with respect to its electrophoretic mobility on Kaltschmidt & Wittmann (1970) gels (cf. Vandrey *et al.*, 1976; Kruiswijk & Planta, 1974). In contrast, studies on the methylation of *Escherichia coli* ribosomal proteins have revealed a much larger number of methylated protein species, which are, moreover, principally present on the large ribosomal subunit (Chang *et al.*, 1974; Chang & Chang, 1974, 1975; Alix & Hayes, 1974).

Similarly to the phosphorylated yeast ribosomal proteins (see Kruiswijk *et al.*, 1978c) the methylated protein components belong to the group of proteins that associate with the immature ribosomal particles at a relatively late stage of ribosome maturation (Kruiswijk *et al.*, 1978a). Therefore we suggest also that the methylation of the yeast ribosomal proteins is not involved in ribosome assembly, but rather may play a functional role in protein biosynthesis.

Our studies demonstrate that the yeast ribosomal proteins L44 and L45, which probably are analogous with the proteins L7 and L12 of *E. coli*, are not methylated *in vivo*. The methylation of L7 and L12 in *E. coli* is still a matter of dispute. Analysis of the primary structure of these closely related proteins revealed that 50% of one particular lysine residue is modified into monomethyl-lysine (Terhorst *et al.*, 1972a,b). On the other hand, Chang & Chang (1975) found a very low degree of methylation of these proteins, whereas Alix & Hayes (1974) could not detect the methylation of these strongly acidic proteins at all.

Analysis of the ribosomal protein hydrolysates of the 40S and 60S subunits demonstrated that in

particular lysine and arginine residues carry methyl groups. With respect to the modified lysine residues it was not possible to distinguish between mono-, di-, and tri-methyl-lysine under the electrophoretic conditions used. Relative to the 60S-subunit proteins, the methylated ribosomal proteins of the 40S subunit contain, relatively, more methylated arginine residues. A similar difference between both types of subunits has been observed in HeLa cells (Chang *et al.*, 1976), whereas in *E. coli* hardly any methylated arginine residues have been found (Chang & Chang, 1975). Recently, the occurrence of α -N-monomethylated methionine and alanine residues in the ribosomal proteins of *E. coli* has been reported (Chen *et al.*, 1977; Chang & Budzilowicz, 1977). Therefore the slightly higher $^3\text{H}/^{14}\text{C}$ ratio of the material on the electrophoretogram migrating in the region of the neutral amino acids (compound X in Fig. 3: $^3\text{H}/^{14}\text{C} = 1.42$) as compared with the $^3\text{H}/^{14}\text{C}$ of methionine (i.e. 1.24) may indicate the presence of methylated neutral N-terminal amino acids in yeast ribosomal proteins.

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