STUDIES OF ODD BASES IN YEAST MITOCHONDRIAL tRNA : III. CHARACTERIZATION OF THE tRNA METHYLASES ASSOCIATED WITH THE MITOCHONDRIA

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SUMMARY : Whereas m^1G, m^2G, m_2^2G, m^7G, T, m^1A, m^5C and Cm methylase activities were found in total cell enzyme of Saccharomyces cerevisiae using undermethylated E. coli tRNA and E. coli B tRNA in reaction with or without Mg^{++}, only m^1G, m^2G, m_2^2G and T methylases occurred in mitochondria. Mitochondrial and cytoplasmic tRNA cannot be methylated by their homologous enzymes ; only mitochondrial tRNA can be methylated in a heterologous reaction by total cell enzyme with formation of T, m^5C, m^1A and low amounts of m^2G and m_2^2G.

INTRODUCTION : Study of the odd bases present in yeast mit. tRNA showed the presence of four distinct methylated bases : T (1,2), m^2G, m_2^2G and m^1G (2). Till now only the occurrence of yeast mit. m^2G and m_2^2G methylases have been reported (3). These results prompted us to characterize the different tRNA methylases present in mit. enzymatic extracts. This report deals with the different methylating activities revealed in mit. and total cell extracts using E. coli B- or undermethylated E. coli tRNA as substrate. We studied also whether it was possible to overmethylate the yeast functional mit.- or cyt. tRNA with homologous or heterologous enzymes.

MATERIALS AND METHODS :

1) Preparation of mit. tRNA from Saccharomyces cerevisiae ρ^+ IL8-8C protoplasts was as in ref. 4. Cyt. tRNA was either total cell tRNA purchased from Boehringer-Mannheim or cyt. tRNA from mit. DNA less mutant IL8-8C/H 71 or from ρ^+ cells IL8-8C, prepared as in (5). E. coli B tRNA was purchased from Boehringer. Undermethylated tRNA was extracted according to Rammler et al (6) from E. coli K 12 58/161 Met^- B_1^- RC^{Rel^-}, starved for methionine during the stationary phase (7). All the tRNAs prepared, were chromatographed on Sephadex G100. The mitochondrial enzyme was prepared from highly purified mitochondria isolated from ρ^+ protoplasts and the total cell enzyme was extracted as earlier described (4,5,8).

2) The 100 µl methylation medium contained Tris-HCl pH 8 10 µmoles, KCl 3 µmoles, $MgCl_2$ 1.5 µmoles, 2-mercaptoethanol 0.5 µmole, glutathione 0.3 µmole, S-adenosyl L methionine (SAM) [^{14}C] CH_3 46 Ci/mole (CEA) 3 nmoles, 700 to 900 µg enzymatic proteins and 4 to 5 µg tRNA. In some reactions we added to this medium 3 µmoles EDTA : these reactions are referred to as "without Mg^{++}". After a two hours incubation at + 37°C an aliquot is applied

Abbreviation : mit. : mitochondrial ; cyt. : cytoplasmic.

on a Whatmann 3 MM paper disk, which is further washed 3 times with cold trichloracetic acid 5%, 2 times with ethanol 95%, dried and counted for radioactivity by liquid scintillation. In the conditions used, a plateau of methylation was reached. For kinetic measurements 200 µg undermethylated E. coli tRNA were methylated for 5 to 40 minutes by 500 µg enzymatic proteins.

3) The analysis of the methylated bases formed with the different enzymes, was performed submitting 1 ml of the above mentioned methylation medium, at the end of the reactions, to phenolic extractions ; the modified tRNA was then purified by Sephadex G25 chromatography, dialysed and dried under vacuum. It was however more convenient to elute the modified tRNA from the disk filters with triethylamine bicarbonate 0.1 M pH 9.7, after removing the scintillator with toluene and ethanol 95%, and drying. The elution was repeated twice and the tRNA was recovered by drying under vacuum.

4) Two dimensional thin layer chromatography on cellulose plates (Schleicher and Schüll G1440) of methylated tRNA formic acid hydrolysates (9) was according to Munns et al (10). The bases were identified by cochromatography of m^1G, m^2G, m^2_2G, m^7G, m^1A, m^2A, m^6A, m^5C, m^1hX, (Cyclochemicals and Sigma), and T formed by formic acid hydrolysis of ribothymidine (Cyclochemicals). The position of the different bases was verified by their alkaline and acidic spectra ; they were essentially similar to those reported in ref. 10. The radioactive spots were detected using one month long autoradiography ; they were then scraped and counted for radioactivity by liquid scintillation. Less than 4% radioactive material was present at the origin.

RESULTS :

1) Overall yield of E. coli tRNA methylation (Table Ia,b) shows that undermethylated E. coli tRNA can be 7 and 4 times more methylated than E. coli B tRNA by respectively mit. and total cell enzymes in the presence of Mg^{++}. Total cell enzyme in reactions "without Mg^{++}", when compared to reactions in the presence of Mg^{++}, methylates more E. coli B tRNA, whereas mit. enzyme gives "without Mg^{++}" a very poor methylation if significant (Table Ia). Measurement of methylase activities (not shown) with undermethylated E. coli tRNA in the presence of Mg^{++} reveals that mit. extracts contain half of methylating activity (5 pmoles CH_3 incorporated per mn and per mg of proteins) compared to total cell extracts (11 pmoles/mn/mg).

2) Methylase activities revealed with E. coli tRNA : Analysis of the methylated bases formed with undermethylated E. coli tRNA in presence of total cell enzyme indicates that besides activities best revealed in the presence of Mg^{++} i.e. T, m^2G, m^2_2G, m^1G methylases, there are activities not or poorly revealed in the presence of Mg^{++}, which appear in reaction "without Mg^{++}" i.e. m^7G, m^1A and m^5C methylases (Table II). Furthermore the methylases revealed with total cell enzyme in presence of Mg^{++} are found in the mit. enzyme whereas those well revealed "without Mg^{++}" are not (m^7G methylase) or very poorly found (m^5C, m^1A methylases) (Table II). It must be noticed that with E. coli B tRNA we found essentially m^2_2G and m^2G methylases in the two enzymatic extracts in the presence of Mg^{++}, and m^1A and m^5C methylases with the total cell enzyme "without Mg^{++}" (not shown). This explains also why total

Table I : Overall yields of various tRNA methylation in presence of total or mitochondrial enzymes with or without Mg^{++}. Results are given in pmoles methyl incorporated per nmole tRNA.

	Total enzyme		Mitochondrial enzyme	
	with Mg^{++}	without Mg^{++}	with Mg^{++}	without Mg^{++}
a. E.coli B tRNA	240	340	75	4
b. E.coli under-methylated tRNA	1060	400	525	90
c. Mitochondrial tRNA	95	14	0	0
d. Cytoplasmic tRNA	< 10	4	0	0

Table II : Base analysis of E. coli undermethylated tRNA and yeast mitochondrial tRNA methylated with total or mitochondrial methylases with or without Mg^{++}. Results are given in percentage of recovered radioactivity.

	E.coli undermethylated tRNA				Mitochondrial tRNA
	Total enzyme		Mitochondrial enzyme		Total enzyme
	with Mg^{++}	without Mg^{++}	with Mg^{++}	without Mg^{++}	With Mg^{++}
m^7G	0	3	0	0	0
m^1G	5	1	6	1.5	0
m^2G	2	1	6.5	1	3
m_2^2G	13	5	8.5	3	1.5
m^5C	< 1	27	0	< 1	7
m^1A	1.5	17	0	< 1	38
T	77	45	79	92	48.5

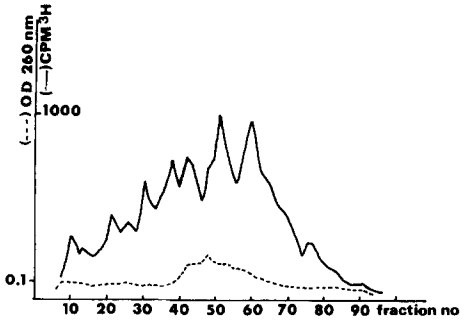

Figure 1 : RPC 5 chromatography (5) of yeast mitochondrial tRNA methylated by total yeast methylases with $[^3H]CH_3$ SAM.

cell enzyme gives increased methylation of E. coli B tRNA "without Mg^{++}" when compared to the reaction with Mg^{++} (Table Ia). Finally we confirmed these results using the nucleosides chromatography according to Rogg et al. (11) (not shown) ; furthermore Cm methylase activity could be revealed in total cell enzyme as already reported (12), whereas no O'-methylases could be detected in mit. enzyme.

3) Homologous and heterologous methylation reactions : no significant methylation could be obtained in homologous reactions (Table Ic, d) with or "without Mg^{++}". We could not methylate cyt. tRNA with mit. enzymes. This was also confirmed by lack of methylation of some purified species of yeast cyt. tRNA : $tRNA^{Phe}$, $tRNA^{Ala}$, $tRNA^{Asp}$, $tRNA^{Arg}_{III}$, $tRNA^{Trp}$ (not shown). However mit. tRNA could be methylated with total cell enzyme specially in the presence of Mg^{++}. We verified by gel electrophoresis that the methyl counts were associated to the peak of tRNA. Several mit. tRNA species are methylated since RPC 5 chromatography of methylated mit. tRNA (experiment performed with $[^3H]$ CH_3 SAM) yielded several peaks (figure 1). The methylated bases formed were essentially T, m^5C and m^1A besides low formation of m^2G and m^2_2G (Table II).

DISCUSSION :

1) The main result of this report is the finding of active m^1G, m^2G, m^2_2G and T methylases in yeast mitochondria. This result is strengthened by : 1- the lack of methylation of normal E. coli B tRNA in reactions "without Mg^{++}" in presence of mit. enzyme, while m^5C and m^1A are formed by the total cell enzyme ; 2- the formation of m^5C and m^1A in mit. tRNA with total cell enzyme. Note that Mg^{++} has opposite effects in the reactions of cyt. m^1A and

m^5C methylases with E. coli B tRNA or mit. tRNA. This may result from differences in tRNA conformations : mit. tRNA would need Mg^{++} so that methylable m^5C and m^1A sites may fit to the enzyme, whereas these sites on E. coli tRNA would not need Mg^{++}. However a m^1A methylase has been characterized in the mitochondria of HeLa cells (13) and in plant mitochondria and chloroplasts (14), thow it is not known if in the latter report chloroplastic and mit. m^1A methylases are different. The presence of a m^1A methylase in higher organisms mitochondria and its absence in yeast mitochondria may be due to evolution of the mitochondrial biosynthesis systems. It must be emphasized that in contrast to Klagsburn's report in the HeLa cells mitochondria (13), we could not find a m^2A methylase in yeast mitochondria.

2) The very low activities of m^5C and m^1A methylases present in mit. enzyme may well be the result of a low contamination by cyt. enzyme since we used conditions where the cyt. m^5C and m^1A methylases are well detected in total cell enzyme. Finally the results are in full agreement with analysis of the methylated bases present in mit. tRNA (2). Studying yeast mit. methylases, Smolar et al (3) found essentially m^2G and m_2^2 G methylases. An inhibitor of methylation might have been present in their crude mit. extracts, which contained mitochondrial membranes and was not purified by Sephadex G25 and DEAE-cellulose chromatography. This would also explain that we could methylate the undermethylated E. coli tRNA 10 times more with our mit. enzyme.

3) Absence of methylation in homologous reactions shows that mit. tRNA as well as cyt. tRNA are fully methylated in vivo. However methylation of mit. tRNA by total cell enzyme is possible. m^5C and m^1A formation can be somewhat expected since the bases are not present in mit. tRNA. The low formation of m^2G and m_2^2G may be due to methylation of incompletely modified sites and may well occur with mit. enzyme also, but would then be too low for detection. This may be an indication that cyt. and mit. m^2G and m_2^2G methylases would be the same enzymes,probably nuclear coded (3). Analysis of the bases in mit. tRNA yielded a little less than 1T per sequence (1,2), however formation of T in this heterologous reaction is rather puzzling because mit. enzyme does not methylate mit. tRNA in vitro despite the presence of an active T methylase. This may be an indication for the existence of distinct enzymes. Work is actually in progress to know whether the mit. methylases and their cytoplasmic counterparts are distinct and if they are nuclear coded.

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