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Purification and Characterization of Nucleolar Ribonucleic Acid Methylase from Ehrlich Ascites Tumor Cells of Mice†

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ABSTRACT: RNA methylases in the extracts of mouse tumor cells were separated on a (diethylaminoethyl)cellulose column in two distinct activities. It was further revealed that the methylase from the isolated nucleoli corresponded to the peak eluted with lower concentration of salt and the extranucleolar nuclear methylase to that with higher salt concentration. Isolation of nucleoli from the purified nuclei was found to be profitable to a 10 times increase of specific activity of nucleolar methylase. The nucleolar methylase has been highly purified and characterized the properties of the enzyme. The purified nucleolar methylase was sedimented as a single component with a molecular weight of 130 000 on a sucrose density gradient. This enzyme is optimally active at pH 7.5 and sensitive to *N*-ethylmaleimide. The presence of a thiol-protecting reagent such as β-mercaptoethanol is necessary for its full activity. The enzyme required no divalent cations and its activity was suppressed in the presence of either 5 mM $MnCl_2$ or 10 mM $MgCl_2$. An apparent K_M value for *S*-adenosyl-L-methionine and a K_I value for *S*-adenosyl-L-homocysteine were 4×10^{-7} M and 1.0×10^{-6} M, respectively. Methyl-acceptance activities of homologous RNAs such as total cellular RNA, nucleolar RNA, and 45S ribosomal precursor RNA of Ehrlich tumor cells by nucleolar methylase from the same tumor cells were negligible, but heterologous RNA, prepared from *Escherichia coli*, served as a good substrate for the nucleolar methylase. On the other hand, hypomethylated RNA was prepared after the treatment of tumor cells with cycloleucine, an inhibitor of *S*-adenosylmethionine formation. The methyl-acceptance activities of both hypomethylated 18S and 28S rRNA were significantly greater than that of hypomethylated tRNA.

In eukaryotic organisms, rRNA precursor is synthesized and methylated in the nucleolus (Greenberg & Penman, 1966). This posttranscriptional modification is confined to rRNA sequences that are conserved during the processing of the precursor RNA (Weinberg et al., 1967). Moreover, all methylation sites within the conserved regions were detected in the specific primary sequence of rRNA (Khan & Maden, 1976). Therefore, elaborate recognition mechanisms may be involved during the modification of rRNA by methylases. Although the in vivo products of rRNA methylation in eukaryotic cells have been extensively studied (Maden & Salim, 1974; Khan et al., 1978), the purification of RNA methylase participating in the modification of ribosomal RNA has not

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been achieved so far. The isolated nucleoli from mammalian cells were merely used to investigate the relationship between synthesis and methylation of nucleolar RNA (Culp & Brown, 1970; Liau et al., 1976).

In this study, we succeeded in separating the RNA methylase activities of nuclei into two distinct fractions, one enriched in the nucleolus and the other in the extranucleolar nuclear fraction. The former enzyme, whose specific activity was about 10 times higher in the isolated nucleoli than in the nuclei, was further purified and characterized by using the heterologous *Escherichia coli* RNAs as a substrate. Moreover, hypomethylated RNAs from tumor cells were prepared to elucidate the specificity of the enzyme after the treatment of these cells with cycloleucine, a competitive inhibitor of ATP:L-methionine *S*-adenosyltransferase (Lombardini et al., 1970). With this hypomethylated RNA, the purified nucleolar enzyme preferably methylated both 18S and 28S rRNAs as compared with tRNA. Both these results and nucleolar localization of the activity suggest that the purified nucleolar enzyme may be involved in the methylation of ribosomal RNA in the nucleolus.

Experimental Procedures

Materials

Chemicals. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (8 Ci/mmol) was purchased from Commissariat à l'Energie Atomique (France). *S*-Adenosyl-L-homocysteine was from P-L Biochemicals, Inc. DEAE-cellulose[1] (DE52) and DEAE-cellulose filter paper disks (DE81) were obtained from Whatman; Sephadex G-150 and poly(U)–Sepharose 4B were obtained from Pharmacia Fine Chemicals, Inc.; Nonidet P-40 was obtained from Shell Chemical Co.; cycloleucine, crystalline bovine serum albumin, catalase, and DNase I were obtained from Sigma Chemical Co. All the other chemicals used were of analytical grade.

Nucleic Acids. Calf thymus DNA was obtained from Sigma Chemical Co. and *Micrococcus luteus* DNA was from Miles Laboratories, Inc. Total cellular RNA from *E. coli* C600 was extracted after the lysozyme digestion (Smith, 1967) and purified by DNase I (10 μg/mL). RNA was routinely extracted by phenol–8-hydroxyquinoline and precipitated with ethanol containing 2% potassium acetate. The fractionation of total *E. coli* RNA into tRNA and 16S and 23S rRNAs was performed by sucrose density gradient centrifugation (McConkey, 1967). In tumor cells, the total RNA and nucleolar RNA were isolated by the sodium dodecyl sulfate–poly(vinyl sulfate)–phenol method (Steele et al., 1965). The 45S rRNA precursor was also prepared from nucleolar RNA by sucrose density gradient sedimentation (Steele et al., 1965).

Methods

Assay for Methylase Activity. The standard reaction mixture (50 μL) contained 50 mM Tris-HCl, pH 7.8, 50 mM ammonium sulfate, 3 mM β-mercaptoethanol, 2 mM EDTA, 15% glycerol, 50 μg of bovine serum albumin, 0.2 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine, and 6 μg of total *E. coli* RNA as the methyl acceptor. After incubation for 15 min at 37 °C, the reaction mixture was chilled in an ice bath, and 45 μL was spotted on a DEAE-cellulose filter paper disk (DE81). The filters were washed 3 times by dipping them in 0.2 M ammonium bicarbonate (Rubin & Modrich, 1977) and then in ethanol–ether (3:1). After the drying of the filter at 80 °C for 10 min in vacuo, the radioactivity was quantitated in toluene-based scintillation fluid (toluene–POPOP–PPO, 1 L:100 mg:8 g).

One unit of methylase activity is defined as 1 pmol of methyl group incorporated into 6 μg of total RNA from *E. coli* during 15 min at 37 °C.

Preparation of Nuclei. Ehrlich ascites tumor cells were grown in the peritoneal cavity of male ddY/F mice and harvested 7 days later. The subsequent isolation of nuclei and nucleoli was carried out as described previously (Higashi et al., 1978). Briefly, the cells were washed with 0.9% NaCl and suspended in hypotonic buffer (10 mM Tris-HCl, pH 7.6, 10 mM KCl, and 1 mM $MgCl_2$) and disrupted in the presence of 0.2% Nonidet P-40 by gentle homogenization. The crude nuclei were sedimented by low-speed centrifugation. The pellets were resuspended in 0.34 M sucrose–6 mM $MgCl_2$ and allowed to stand for 5 min at 0 °C. Thereafter, nuclei were sedimented through a 0.88 M sucrose layer by centrifugation at 2000*g* for 10 min. For further fractionation of nuclei, the pellets were suspended in 0.28 M sucrose–0.3 mM $MgCl_2$.

Fractionation of Nuclei into Subfractions. Nucleoli were isolated from nuclei by the sonication procedure (Busch, 1967). A suspension of nuclei was sonicated by a Branson sonifier (Model B-12) until no unbroken nuclei were detected by phase-contrast microscopy. The resulting sonicate was layered over equal volumes of 0.88 M sucrose and centrifuged at 2000*g* for 20 min. Purified nucleoli were obtained as pellets, while the upper layer containing 0.28 M sucrose–0.3 mM $MgCl_2$ was pooled to use as an enzyme source of extranucleolar nuclear fraction. When 20 fields of the nucleolar preparation were randomly examined by phase-contrast microscopy after the staining of nucleoli (>3000) with Azure C, nuclei were not observed.

Preparation of Extracts from Nuclei, Nucleoli, and Extranucleolar Nuclear Fractions. The nuclei and nucleoli isolated were separately suspended in the solubilization buffer consisting of 50 mM Tris-HCl, pH 7.8, 50 mM ammonium sulfate, 2 mM EDTA, 3 mM β-mercaptoethanol, 15% glycerol, and 0.01% Triton X-100 (buffer A) by a homogenizer. The resulting suspension was sonicated with a sonifier-equipped microtip for 60 s and allowed to stand for 15 min in an ice bath under stirring. The crude extracts obtained were centrifuged at 105000*g* for 35 min in a 50 Ti rotor (Beckman). The clarified supernatant was saved for further purification. On the other hand, extranucleolar nuclear fractions were directly ultracentrifuged to obtain the clarified extracts after separation from the nucleoli, as described in the preceding section.

Separation of Enzyme Activities of Extracts on DEAE-cellulose Column. Nuclear extracts were directly loaded onto a DEAE-cellulose column (1.2 × 4.5 cm), which had been equilibrated with 50 mM Tris-HCl, pH 7.8, 50 mM ammonium sulfate, 2 mM EDTA, 3 mM β-mercaptoethanol, and 15% glycerol (buffer B). Extranucleolar nuclear extracts were mixed with 0.5 volume of 3 times concentrated buffer B to collect the methylase in the extracts by the following batchwise adsorption method. Either the nucleolar extracts or the diluted extranucleolar nuclear extracts were mixed with 4 g (wet weight) of DEAE-cellulose equilibrated with buffer B. After the mixtures were stirred for 30 min at 2 °C, DEAE-cellulose

[1] Abbreviations: Ado-Met, *S*-adenosyl-L-methionine; Ado-Hcy, *S*-adenosyl-L-homocysteine; Bes, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; DEAE, diethylaminoethyl; Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PPO, 2,5-diphenyloxazole; POPOP, 2,2′-(*p*-phenylene)bis(5-phenyloxazole); Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane.

having adsorbed proteins was collected by centrifugation, resuspended in buffer B, and packed into the same size column as used for nuclear preparation. After the column was washed with 5 bed volumes of buffer B, the proteins were eluted with 30 mL of a linear gradient from 0 to 0.3 M NaCl in buffer B at a flow rate of 6.6 mL/h. Fractions (1.25 mL) were collected and aliquots were assayed for enzyme activity under standard conditions.

Purification of Nucleolar Methylase. All steps for the purification of the enzyme were carried out at 0–4 °C unless otherwise indicated. Nucleolar methylase, except for the comparisons of the profiles of methylase on the DEAE-cellulose column as described in the preceding section, was usually purified as follows.

(*A*) *Ammonium Sulfate Precipitation.* The nucleolar extracts prepared from Ehrlich ascites tumor cells [(1–2) × 10^{10} cells] were fractionated with ammonium sulfate precipitation. Solid ammonium sulfate was added to achieve 65% saturation under stirring for 30 min at 2 °C. The resulting suspension was centrifuged at 10000*g* for 10 min and the pellets were dissolved in 0.9 mL of buffer B. Insoluble materials in this solution were removed by centrifugation at 10000*g* for 10 min. The clarified supernatant was used for the next gel filtration of the enzyme. About 85% of the enzyme activities of nucleolar extracts were recovered in this preparation.

(*B*) *Sephadex G-150 Column Chromatography.* The enzyme, after ammonium sulfate fractionation, was applied to a Sephadex G-150 column (1.5 × 20 cm) preequilibrated with buffer B. The gel filtration was carried out in an ascending direction with the same buffer at a flow rate of 5.1 mL/h. Fractions (0.85 mL) were collected and their enzyme activities were assayed. The fractions containing methylase activities were pooled.

(*C*) *DEAE-cellulose Column Chromatography.* The filtrate of Sephadex G-150 was loaded onto a DEAE-cellulose column (1.2 × 8 cm) equilibrated with buffer B. After the column was washed with 3 bed volumes of buffer B, the elution was performed with 40 mL of a linear gradient from 0 to 0.3 M NaCl in buffer B at a flow rate of 6 mL/h.

(*D*) *Poly(U)–Sepharose 4B Column Chromatography.* Fractions containing RNA methylase activity from a DEAE-cellulose column were pooled, adjusted to 0.01% Triton X-100, and applied onto a column (1.2 × 2 cm) of poly(U)–Sepharose 4B that had been equilibrated with buffer A. After the column was washed with 15 mL of buffer A, the proteins bound to the column were eluted with 20 mL of a linear gradient from 0.1 to 0.6 M NaCl in buffer A at a flow rate of 9.6 mL/h. The pooled fractions with high specific activity were mixed with equal volumes of 80% glycerol and stored at −80 °C until further use. Under these conditions, the enzyme preparation was stable over several months.

Preparation of Hypomethylated RNAs from Mouse Tumor Cells after Cycloleucine Treatment. The procedures to obtain hypomethylated RNA from cultured cells were essentially similar to those reported by Caboche & Bachellerie (1977). Ehrlich tumor cells harvested from the peritoneal cavity of mice 7 days after inoculation were collected by low-speed centrifugation and suspended (1 × 10^6 cells/mL) in the modified Eagle's medium (Eagle, 1959), in which the concentration of methionine was reduced to 10 μM and supplemented with Bes–Tes–Hepes buffer, pH 7.0 (Eagle, 1971), 1 mM each of nonessential amino acids, and calf serum (2%). The cell suspension was kept at 37 °C for 2 h, and then cycloleucine was added to the final concentration of 1 mg/mL. After 1.5 h, the cells were harvested by centrifugation and

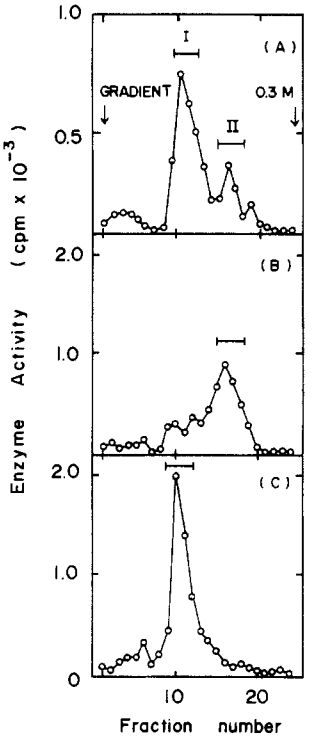

FIGURE 1: Subnuclear distribution of methylase. Purified nuclei were separated into nucleoli and extranucleolar nuclear fractions. The high-speed centrifugation supernatant of nuclear (A), extranucleolar nuclear (B), and nucleolar (C) fractions was chromatographed with a DEAE-cellulose column and assayed for methylase activity (O) under the same conditions as described in the text.

washed with 0.9% saline solution. The extraction of cytoplasmic RNAs and fractionation of the RNA in sucrose density gradients were carried out as described above.

Sucrose Density Gradient Centrifugation. Samples (200 μL) with either catalase or bovine serum albumin were layered on 4.0 mL of linear 5–20% sucrose gradients prepared in buffer B and centrifuged in a Beckman SW 50.1 rotor at 40000 rpm for 12 h at 2 °C. Fractions (130 μL) were collected from the bottom of the tube and assayed for methylase activity. The position of marker proteins was determined by measuring the absorbance at 280 nm.

Protein Determination. Proteins were quantitated by the method of Kalb & Bernlohr (1977).

Results

Distribution of Methylase in Subnuclear Fractions. So that the distribution of methylase in the nuclei could be examined, the extracts from nuclei, nucleoli, and extranucleolar nuclear fractions were prepared as described under Experimental Procedures, adsorbed to a DEAE-cellulose column, and eluted under the same conditions. The results are shown in Figure 1. Methylases in the nuclear extracts were separated on a DEAE-cellulose column into two distinct activities (Figure 1A); that is, one enzyme peak was eluted at about 0.1 M NaCl and the other was eluted at about 0.2 M NaCl, which are tentatively designated as methylases I and II, respectively. On the other hand, the fractionation of nuclei into subfractions, which was prior to chromatography, resulted in the single peak of methylase corresponding to one of these two distinct activities on a DEAE-cellulose column. It was evident from the comparison of elution profiles (Figure 1B,C) that the main peaks of methylase in the extracts of nucleoli and in those of extranucleolar nuclear fractions corresponded to nuclear methylase I and methylase II, respectively. This is the first demonstration that nucleolar methylase can be chromato-

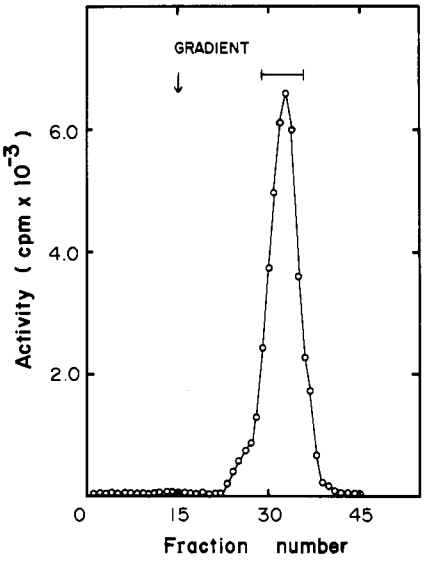

FIGURE 2: Purification of nucleolar methylase by poly(U)–Sepharose 4B column chromatography. The pooled fractions containing methylase activities from the DEAE-cellulose column were applied to a column of poly(U)–Sepharose 4B. The elution was performed with a 20-mL linear gradient from 0.1 to 0.6 M NaCl in buffer A. Fractions (670 μL) were collected and the aliquots were assayed for enzyme activity (O) under standard conditions.

graphically distinguished from extranucleolar nuclear methylases.

Purification of Nucleolar Enzyme. Nucleoli were suspended in solubilization solution (buffer A) and disrupted by sonication. More than 90% of the total methylase activities in this sonicate were recovered in the supernatant after centrifugation (see Experimental Procedures). They were fractionated with ammonium sulfate precipitation, and the enzyme was purified by column chromatography on Sephadex G-150, DEAE-cellulose, and poly(U)–Sepharose 4B (Figure 2).

A summary of the purification scheme is presented in Table I. The isolation of nucleoli from nuclei resulted in about 10-fold purification of nucleolar methylase. In addition to this purification, it was noteworthy that poly(U)–Sepharose 4B column chromatography was very effective for removing the associating proteins in the fractions of the preceding column chromatography. Ultimately, nucleolar methylase I was purified more than 4700-fold over the nuclear fractions.

The final nucleolar methylase preparation was electrophoresed on a 7.5% polyacrylamide gel without sodium dodecyl sulfate and stained by the ultrasensitive silver method (Oakley et al., 1980). It was observed that one major band consisted of more than 85% of staining intensity after densitometric determination (not shown here). Due to extremely low protein concentration of purified enzyme preparation even in the large scale purification, we did not attempt further purification.

Under standard assay conditions, methylase activity was found to be proportional to the amounts of purified enzyme and *S*-adenosyl-L-methionine added. By using this enzyme preparation, we examined general properties of nucleolar methylase I as described below.

Molecular Weight. Highly purified methylase I was ultracentrifuged in a sucrose gradient and sedimented as a single component (Figure 3). A molecular weight of 130 000 was estimated by the method of Martin & Ames (1961).

Optimum pH. Methyltransferase activity of methylase I was assayed at pH 6.0–9.0 under standard conditions. The pH dependency of the reaction was shown in Figure 4. Neither significant inhibition nor stimulation by different buffer systems used was observed. This enzyme was rather

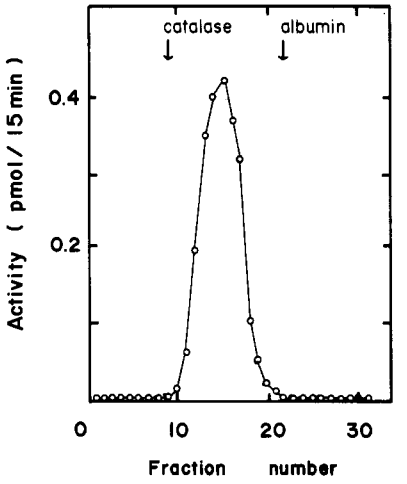

FIGURE 3: Sucrose density gradient centrifugation of nucleolar methylase. Purified nucleolar methylase and protein markers were centrifuged in a 5–20% sucrose gradient as described in the text. Fractions were collected from the bottom of the tube and assayed for methylase activity. The arrows indicate the sedimentation position of marker proteins analyzed on parallel gradients.

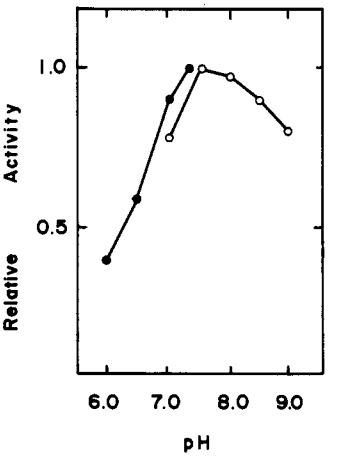

FIGURE 4: Effect of pH on nucleolar methylase activity. Methylase activity of purified enzyme preparation was assayed at pH 6.0–9.0 at 37 °C. Buffers used were 50 mM phosphate buffer (●) for pH 6.0–7.4 and 50 mM Tris-HCl buffer (O) for pH 7.0–9.0. Because Ado-Met is very unstable in the alkaline range (Parks & Schlenk, 1958), the enzyme activity was not tested above pH 9.0.

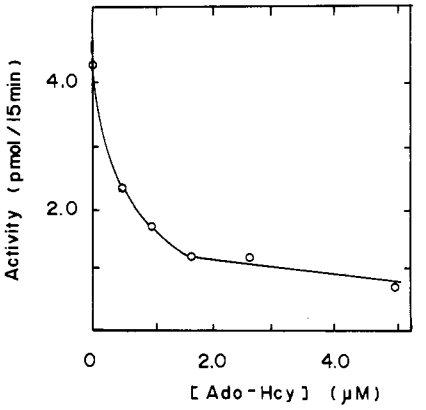

FIGURE 5: Inhibition of transmethylation by *S*-adenosyl-L-homocysteine. At different concentrations of Ado-Hcy, methylase activity was assayed in the presence of 0.6% Me_2SO (O).

active at the alkaline range tested and showed optimum pH at about 7.5.

Kinetic Parameters. At steady-state conditions, the initial reaction velocities were measured at six different concentra-

Table I: Summary of Purification of Nucleolar Methylase from Ehrlich Ascites Tumor Cells[a]

purification step	total protein (mg)	total act. (units)[b]	sp act. (units/mg)	x-fold
nuclei	685	4.97×10^4	7.26×10	1
extract of isolated nucleoli	32.2	2.45×10^4	7.61×10^2	10.5
Sephadex G-150 column chromatography	8.79	1.04×10^4	1.18×10^3	16.3
DEAE-cellulose column chromatography	0.49	4.36×10^3	8.90×10^3	123
poly(U)-Sepharose 4B column chromatography	0.01[c]	3.46×10^3	3.46×10^5	4766

[a] The purification procedure is described in detail under Experimental Procedures. [b] A unit of enzyme activity is defined as 1 pmol of methyl group transferred to total *E. coli* RNA in 15 min under the standard assay conditions described in the text. [c] As the protein concentrations were too low to determine precisely, a limit value of the quantitation method employed was indicated.

Table II: Effects of Sulfhydryl-Binding Reagent and Metal Ions[a]

addition	concn (mM)	methylation (%)
none		100
$MgCl_2$	1.0	102
$MgCl_2$	2.0	98
$MgCl_2$	3.0	103
$MgCl_2$	5.0	39
$MgCl_2$	10	5
$MnCl_2$	1.0	93
$MnCl_2$	2.0	97
$MnCl_2$	3.0	25
$MnCl_2$	5.0	3
N-ethylmaleimide	0.5	84
N-ethylmaleimide	1.0	60
N-ethylmaleimide	2.0	45
N-ethylmaleimide	5.0	2

[a] Standard assay conditions were used. Methylation is expressed as percentage relative to the level of methylation in the absence of the chemicals listed.

tions of Ado-Met. An apparent K_M value was calculated from a $1/v$ vs. $1/[\text{Ado-Met}]$ plot (Lineweaver & Burk, 1934). The effect of Ado-Hcy, a reaction product derived from Ado-Met, on the enzyme activity was also tested at different concentrations. Figure 5 shows the result. From a similar linear plot, this type of inhibition was found to be the competitive one. An apparent K_M value for Ado-Met and a K_I value for Ado-Hcy were 4×10^{-7} M and 1.0×10^{-6} M, respectively.

Effects of Sulfhydryl-Binding Reagent and Metal Ions. Under standard assay conditions, various concentrations of chemicals such as *N*-ethylmaleimide, $MgCl_2$, and $MnCl_2$ were separately added into the incubation mixture for methylase I at 37 °C for 5 min before the addition of *S*-adenosyl-L-[*methyl*-^{3}H]methionine. Thereafter, the remaining activity was determined under standard assay conditions. Divalent cations were not required for the enzyme activity. Namely, no stimulation of activity was observed in the range of concentration from 1 to 3 mM cations; besides, the methylase activity was severely inhibited at higher concentrations of these ions, that is, 5 mM Mn^{2+} and 10 mM Mg^{2+} (Table II). Dose-dependent inhibition of activity was observed for *N*-ethylmaleimide. At 5 mM *N*-ethylmaleimide, complete inhibition was achieved. Furthermore, reducing reagents such as β-mercaptoethanol were essential for the full activity of nucleolar methylase. Thus, these results suggest that a free active thiol group(s) of enzyme may be involved in the methyl-transferring reaction.

Substrate Specificity. RNAs and DNAs from various sources were examined. Their methyl-acceptance activities were determined as described under Experimental Procedures.

Table III: Substrate Specificity of Purified Nucleolar Methylase[a]

acceptors	methyl group incorporated (cpm)
E. coli total cellular RNA	6070
Ehrlich ascites tumor cells	
total cellular RNA	146
nucleolar RNA	130
45S rRNA precursor	78
calf thymus DNA	98
M. luteus DNA	103

[a] Methyl-acceptance activity of nucleic acids indicated was determined under standard assay conditions in which 6 μg of each nucleic acid and 10 units of nucleolar enzyme were employed.

Table IV: Methylation of Heterologous RNA from *E. coli* and Hypomethylated Homologous RNA from Mouse Tumor Cells by Purified Nucleolar Methylase in Vitro[a]

(A) heterologous	enzyme act. (cpm)	(B) hypomethylated[b]	enzyme act. (cpm)
tRNA	5500 (1.0)	tRNA	312 (1.0)
16S rRNA	2216 (0.4)	18S rRNA	1440 (4.6)
23S rRNA	1116 (0.2)	28S rRNA	2060 (6.6)

[a] Standard assay conditions as described under Experimental Procedures were used. The reaction mixture contained 6 μg of each RNA indicated and 5 units for (A) and 3 units for (B) of the enzyme. [b] These substrates were prepared from mouse tumor cells after the treatment with cycloleucine (see Experimental Procedures). The values in parentheses indicate the ratio of methyl-acceptance activity of each rRNA to tRNA.

These results were summarized in Table III. Although DNAs from *M. luteus* and calf thymus had been generally used as a substrate for the DNA methylase from several mammalian cells (Sneider et al., 1975; Adams et al., 1979), purified methylase I scarcely transfers methyl groups to these DNAs. Therefore, we consider that purified nucleolar enzyme preparations are free from DNA methylase activities. It was also shown that all of the homologous RNAs tested, which were prepared from the same mouse tumor cells as the enzyme, had essentially no methyl-acceptance activities for methylase I. Increased amounts of these RNAs gave similar results (data not shown). On the contrary, heterologous RNAs from *E. coli* served as good substrates for nucleolar methylase (Table III). In the next place, RNAs were fractionated by sucrose density gradient centrifugation and their methyl-acceptance activities were examined (Table IV). In the experiments with heterologous RNA, incorporation of methyl groups into tRNA of *E. coli* was greater than that of rRNA of *E. coli*, although the extent of methyl-group transfer to the rRNA of *E. coli* was significantly higher than those values with normally methylated rRNA from mouse tumor cells (cf. Table III).

However, as expected from the origin of the enzyme, the nucleolar methylase transferred 5–7 times more methyl groups to both 18S and 28S rRNA than to tRNA obtained from the tumor cells treated with cycloleucine.

Discussion

It is well-known that the nucleolus is the site of synthesis and methylation of rRNA precursors. In rapidly growing cells such as Ehrlich ascites tumor cells, the methylation activity in the nucleoli must be high due to the increased production of ribosomes in this organelle. Actually, the nucleoli of Ehrlich ascites tumor cells are generally large sized and are easily isolated by the method employed routinely in our laboratory (Muramatsu et al., 1963; Higashi et al., 1978). The purity of isolated nucleoli is adequate to analyze the nucleolar components, which are essentially free from the extranucleolar contaminants. From these nucleoli, the RNA methylase activities have been successfully solubilized and extensively purified in this work.

The localization of methylase I in the nucleoli was suggested from the following two results: first, nucleolar extracts had approximately 10-fold higher specific activity over the nuclear extracts (Table I) and, second, the methylase I solubilized from the isolated nucleoli could be distinguished from extranucleolar nuclear enzyme(s) (methylase II) by the pattern of DEAE-cellulose column chromatography (Figure 1). This paper showed the first demonstration of nucleolus-specific RNA methylase, which was highly purified from the extracts of the isolated nucleoli, although the endogenous activities of RNA methylation were reported previously in the isolated nucleoli by others (Culp & Brown, 1970; Liau et al., 1973).

An apparent K_M value of methylase I for Ado-Met was 4×10^{-7} M, which is comparable with the previous values for either tRNA methylases or DNA methylases. Namely, K_M values for Ado-Met of tRNA methylases were reported from 1.5×10^{-6} to 7×10^{-5} M (Salvatore et al., 1979) and those of DNA methylases ranged from 2.5×10^{-6} (Simon et al., 1978) to 1.3×10^{-5} M (Turnbull & Adams, 1976). Furthermore, the present methylase I from the nucleoli shares several properties with the previous enzymes for methylation of either tRNA or DNA (Salvatore et al., 1979; Simon et al., 1978). In the alkaline range, the enzymes are optimally active; reducing agents such as β-mercaptoethanol are required for their full activity; Ado-Hcy is a potent inhibitor for all of these methylases. The effect of magnesium ions on the methylase activities was, however, different from one to the other; that is, nucleolar methylase I was strongly inhibited by various concentrations of $MgCl_2$ while tRNA methylases from several eukaryotes were reported to be stimulated by magnesium ions (Salvatore et al., 1979). DNA methylases did not show any sensitivity for such ions (Turnbull & Adams, 1976; Rubin & Modrich, 1977; Simon et al., 1978; Adams et al., 1979).

In the nucleoli, the transcribed spacer regions of rRNA precursor were considered not to be methylated (Weinberg et al., 1967), while most methylated sequences occurred only once in a molecule of mature rRNA in a *Xenopus laevis* (Khan & Maden, 1976; Khan et al., 1978). Accordingly, methylases involved in the modification of rRNA precursors can recognize specific structures, including base sequences, configurations of RNA, and its associated proteins. Homologous RNA, which was prepared from the same sources as the methylase, would be a poor methyl acceptor presumably because those methylation sites have been saturated in a specific fashion in vivo. Actually, purified nucleolar methylase I did not transfer methyl groups to such homologous RNAs, including 45S rRNA precursor (Table III); that is, nonspecific methylation of these RNAs by methylase I hardly occurred under the present conditions. On the other hand, we observed that heterologous rRNA from *E. coli* was a good substrate as a methyl acceptor, although more methyl groups were transferred to the tRNA of *E. coli* (Table III). Therefore, nucleolar methylase has sequence specificity since it does not methylate homologous RNAs but does methylate *E. coli* RNAs. In other words, this enzyme recognizes precise sequences that are serving as sites for methylation.

To examine the substrate specificity of purified nucleolar methylase under more physiological conditions, we prepared hypomethylated RNAs after the treatment of mouse tumor cells with cycloleucine, by which both rRNA and tRNA in CHO cells were significantly undermethylated (more than 90%) (Caboche & Bachellerie, 1977; Amalric et al., 1977). Under standard assay conditions, both hypomethylated 18S and 28S rRNA were 5–7 times more methylated by the purified nucleolar enzyme than the tRNA from the same cells (Table IV). These differences of methylation of homologous RNAs obtained from tumor cells with and without the drug treatment further suggest the high substrate specificity of purified nucleolar methylase as regards to the methylation site(s).

So far we have not characterized the precise sequences serving as sites for methylation with the hypomethylated nucleolar precursor of rRNA. However, nucleolar localization of this enzyme and high substrate specificity with hypomethylated rRNA strongly suggest that methylase I participates in the modification of rRNA in the nucleoli. For elucidation of this possibility, the analysis of the products in vitro might be useful, and this is now under investigation in our laboratory.

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Reexamination of the Binding Site for Pyridoxal 5′-Phosphate in Ribulosebisphosphate Carboxylase/Oxygenase from *Rhodospirillum rubrum*†

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ABSTRACT: The high specificity of pyridoxal 5′-phosphate (PLP) for an essential lysyl residue of ribulosebisphosphate carboxylase/oxygenase was confirmed, but half-of-sites reactivity was not observed in contrast to an earlier report [Robison, P. D., Whitman, W. B., Waddill, F., Riggs, A. F., & Tabita, F. R. (1980) *Biochemistry 19*, 4848–4853]. Subsequent to reduction with [^{3}H]borohydride and tryptic digestion of the enzyme inactivated by PLP, the sole labeled peptide was purified by successive chromatography on DEAE-cellulose, SP-Sephadex, and Sephadex G-25. The peptide, recovered in good yield, appeared essentially homogeneous by amino acid analysis, peptide mapping, and sequencing. Automated Edman degradation established the peptide's sequence as Val-Leu-Gly-Arg-Pro-Glu-Val-Asp-Gly-Gly-Leu-Val-Val-Gly-Thr-Ile-Ile-(PLP)Lys-Pro-Lys instead of Ala-Leu-Gly-Arg-Pro-Glu-Val-Asp-(PLP)Lys-Gly-Thr-Leu-Val-Ile-Lys as reported by Robison et al. (1980) [Robison, P. D., Whitman, W. B., Waddill, F., Riggs, A. F., & Tabita, F. R. (1980) *Biochemistry 19*, 4848–4853]. The sequence -Ile-Lys-Pro-Lys- in the former is identical with that encompassing Lys-175 in the carboxylase/oxygenase from spinach, which reacts preferentially with PLP and two other affinity labels. This finding of homology greatly strengthens the supposition that Lys-175 in the spinach enzyme and the corresponding lysyl residue in the *Rhodospirillum rubrum* enzyme are active-site residues and furthermore increases the likelihood of their functionality in catalysis.

Three different affinity labels for D-ribulose 1,5-bisphosphate (ribulose-P_2)[1] carboxylase/oxygenase from spinach, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate (Stringer & Hartman, 1978), *N*-(bromoacetyl)ethanolamine phosphate (Schloss et al., 1978), and PLP (Paech & Tolbert, 1978; Spellman et al., 1979), implicate the same lysyl residue as an active-site component. Based on the primary structure deduced from the sequence of the gene for the carboxylase, this lysyl residue occupies position 175 (McIntosh et al., 1980; Zurawski et al., 1981). Although catalytic functionality of the lysyl ϵ-amino group has not been proven, its unusual reactivity is consistent with an essential role (Norton et al., 1975). Comparative sequence studies are useful to discern residues involved in catalysis because of conservation of essential molecular features during evolution. Comparisons between the carboxylase from spinach and *Rhodospirillum rubrum* appear especially appropriate because these organisms are evolutionarily quite distant (McFadden & Tabita, 1974), and the two enzymes differ markedly in both primary and quaternary structure (Siegel et al., 1972; Tabita & McFadden, 1974). Total sequence homology is probably less than 20% (Akazawa et al., 1978; Stringer et al., 1981).

Of the three aforementioned affinity labels, only PLP has been thoroughly investigated in its reaction with ribulose-P_2 carboxylase from *R. rubrum* (Whitman & Tabita, 1978a,b). Since several criteria for affinity labeling were fulfilled, the reported lack of sequence homology of the phosphopyridoxal

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[1] Abbreviations: ribulose-P_2, D-ribulose 1,5-bisphosphate; PLP, pyridoxal 5′-phosphate; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Quadrol, *N*,*N*,*N*′,*N*′-tetrakis(2-hydroxypropyl)ethylenediamine; TPCK, tosylphenylalanyl chloromethyl ketone.

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