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Partial Purification and Characterization of an N^2-Guanine RNA Methyltransferase from Chicken Embryos†

Paola Izzo‡ and Raymond Gantt*

ABSTRACT: An N^2-guanine RNA methyltransferase has been purified 1000-fold from chick embryo homogenates by phosphocellulose chromatography followed by chromatography on *S*-adenosylhomocysteine-Sepharose. The enzyme was shown to methylate the G_{10} position of *Escherichia coli* B $tRNA^{Phe}$ and has a K_m of 3×10^{-7} M for $tRNA^{Phe}$ and 1.38×10^{-6} M for *S*-adenosylmethionine. The molecular weight was estimated to be 77 000 by gel filtration and the pH optimum was 8.0 to 8.5. Magnesium ion was not required for activity but it stimulated the rate of methylation 1.5-fold with an optimum at 12 mM. Ammonium ion stimulated activity about twofold with an optimum at about 83 mM. Sodium and potassium ions above 0.1 M were inhibitory.

Many enzymes in cells are involved in nucleic acid modification. The methylation of nucleosides in various positions and combinations is the most common modification and in the transfer RNA of some eukaryotes 5 to 10% of the bases may be modified by enzymatic methylation. The other major fractions of nucleic acids are also methylated including DNA, messenger RNA, 18S and 28S ribosomal RNA, and small molecular weight nuclear RNA (for reviews, see Borek and Srinivasan, 1966; Starr and Sells, 1969; Weinberg, 1973). The biologic function(s) of these diverse reactions are generally unknown, though a few have been discovered. For example, 6-methylaminopurine and 5-methylcytosine in bacterial DNA have been shown to prevent cleavage of DNA at specific sites by restriction enzymes (Arbor, 1974), and the 7-methylguanine at the 5′-phosphate end of messenger RNA has been shown (Muthukrishnan et al., 1975) to be a requirement for translation in some cases though not all (Rose and Lodish, 1976).

Two technical problems have hindered the study of this group of enzymes. First, the high specificity of the enzymes for a particular base, position on the base, and the structure around

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the base necessitates the use of very special substrates (in some cases actually not available), and either partially purified enzyme preparations or complex separation procedures are required to ascribe the measurement of a given methyl group incorporated to a single enzyme. Second, these enzymes tend to be unstable resulting in large losses of activity during purification procedures. To our knowledge the few reports concerned with purifying N^2-guanine RNA methylases from animal cells (Kuchino and Nishimura, 1970; Agris et al., 1974; Kraus and Staehelin, 1974a,b; Glick et al., 1975) have purification factors of less than 200-fold. In this report we have partially characterized and purified an N^2-guanine RNA methyltransferase from chick embryo cells 1000-fold by methods which may be generally useful for nucleic acid methylases.

Materials and Methods

Enzyme Source. Twenty 10-day-old chick embryos were harvested, washed twice in 0.01 M phosphate-buffered saline (pH 7.2), and homogenized in a glass homogenizer with a motor-driven Teflon pestle with 30 mL of buffer A [30 mM Tris-HCl (pH 7.5), 20 mM NaCl, 1 mM EDTA,[1] 2.8 mM β-mercaptoethanol, 10% glycerol (w/v)]. The homogenate was centrifuged at 20 000*g* for 30 min, and the supernatant was centrifuged, again, at 105 000*g* for 90 min. This high-speed supernatant was routinely shell frozen in 20-mL aliquots in dry ice–ethanol and stored at −20 °C.

Enzyme Assay. The guanine RNA methyltransferase activity was measured as radioactive RNA precipitated on glass fiber disks. The standard incubation mixture contained 75 mM Tris-HCl (pH 8.9), 6.6 mM magnesium chloride, 13.3 mM β-mercaptoethanol, 4.28 μM *S*-adenosylmethionine (1.7 μCi, sp act. 6.79 Ci/mmol, New England Nuclear), 0.33 mg/mL *Escherichia coli* B sRNA (Calbiochem) or commercially purified tRNAs (Miles Laboratories), and 5 to 20 μL of enzyme source in a total volume of 60 μL. After 30 min of incubation at 37 °C the reaction was stopped by the addition of 3 mL of cold 10% trichloroacetic acid. The precipitate was collected on a glass fiber filter (type A-E, 25 mm diameter, Gelman Instrument Corp.) and washed four times with 10 mL of cold 10% trichloroacetic acid and four times with 10 mL of 95% ethanol. The filters were dried for 10 min at 100 °C and counted in 10 mL of Econofluor (New England Nuclear) in a Beckman LS-250 liquid scintillation counter. Complete incubations precipitated at zero time were used as blanks. Protein concentration was determined by the method of Lowry et al. (1951) or absorbance at 280 nm.

Identification of Methylated Products. The assay of individual methylated bases was determined by high-resolution pressure chromatography when enzyme activities other than N^2-guanine methylase might be present. The procedure is essentially as described earlier (Gantt et al., 1975). The glass fiber disk of a methylase assay, before or after counting, is air dried and hydrolyzed at 100 °C for 30 min in 1 N HCl. A 0.5-mL aliquot containing added methylated base standards was applied to a 5 mm × 38 cm column of Beckman AA-15 cation-exchange resin. Column flow rate was maintained at 1 mL/5 min with variable pressure (270–300 psi). The column was equilibrated and eluted with buffer containing 1 L of ammonium acetate (1 M), 350 mL of glacial acetic acid, and 50 mL of water (pH 4.1). The eluate was monitored at 254 nm. Fractions (1 mL) were counted in 15 mL of Aquasol fluor (New England Nuclear) containing 5% water. Tritium was monitored at 35% efficiency under these conditions.

Affinity Chromatography. Activated CH-Sepharose 4B (Pharmacia) (15 g) was suspended in 100 mL of 1 mM HCl and washed with 3 L of 1 mM HCl on a sintered glass filter. SAH (Sigma Chemical Co.) (300 mg) was dissolved in 75 mL of 0.1 M $NaHCO_4$ (pH 8.0) and mixed with the gel suspension in an end-over-end mixer for 1 h at room temperature. The excess ligand was washed away with coupling buffer on a sintered glass filter and the remaining reactive groups were blocked with 1 M ethanolamine (pH 9.0). The SAH–Sepharose was then washed with three cycles of buffers at high pH (0.05 M Tris-HCl (pH 8.0), 1 M NaCl) and low pH (0.05 M formate (pH 4.0), 1 M NaCl) and finally equilibrated with buffer B [75 mM Tris-HCl (pH 8.5), 6.6 mM magnesium chloride, 13.3 mM β-mercaptoethanol, 1 mM EDTA, 10% glycerol (w/v)].

The amount of SAH bound to the Sepharose was determined by measuring the adenine released by acid hydrolysis. One milliliter of SAH–Sepharose was mixed with 1 mL of 1 N HCl and heated at 100 °C for 30 min. The hydrolysate (0.5 mL) was chromatographed as described above for assay of methylated bases except no base standards were added. One-milliliter fractions were collected and the adenine peak was measured at A_{260} and the concentration calculated. Six to eight micromoles of SAH bound to 1 mL of Sepharose.

Neutral Sodium Dodecyl Sulfate Gel Electrophoresis. A 5% acrylamide gel slab (10 cm × 10 cm × 0.1 cm) was formed similar to that of Maizel (1971) at pH 7.1. The protein samples were prepared by precipitation from solution with cold 10% trichloroacetic acid and washing the precipitate with acetone to remove the acid. The residue was dissolved in 20 μL of a solution containing 1% sodium dodecyl sulfate, 0.01 M sodium phosphate, 1% (v/v) β-mercaptoethanol, and 10% (w/v) glycerol and heated for 10 min at 100 °C. Electrophoresis was performed at 50 mA for 6–7 h. The gel slab was stained in 0.25% Coomassie Brilliant Blue R-250 in 50% trichloroacetic acid for 1 h, followed by repeated washes in 7% acetic acid to remove the unbound stain.

Results

Purification. One general approach to rapid purification of RNA methylases is the procedure used widely for other proteins where a general, preliminary, separation technique such as ion exchange chromatography is followed by affinity chromatography. To do this we chose to start with an anion exchange column (Perffetto and Izzo, 1974) followed by chromatography on an SAH–Sepharose column. Figure 1 shows the elution from a phosphocellulose column of a frozen high-speed supernatant of an embryo cell homogenate. Analysis of the enzymatic activity of the first peak which washed through the column showed various bases containing radioactivity including methyladenines, methylated pyrimidine nucleotides, and methylated guanines including N^2-methylguanine. The second radioactive peak contained only N^2-methylguanine (Figure 2). To test whether the N^2-guanine methylase activity in the first peak came through the column because of overloading, the fractions were combined and passed through a second column and all the activity again came through, which indicates the two activities are different enzymes. The purification factor with this column is 39-fold.

After precipitating fractions 62–65 (Figure 1) with 75% ammonium sulfate, resuspending the precipitate in buffer B, and dialyzing against buffer B for 8 h to remove residual am-

[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SAH, *S*-adenosylhomocysteine; hu, dihydrouridine; DEAE, diethylaminoethyl; T_1 RNase, Takadiastase ribonuclease 1 or guanyloribonuclease (EC 3.1.4.8).

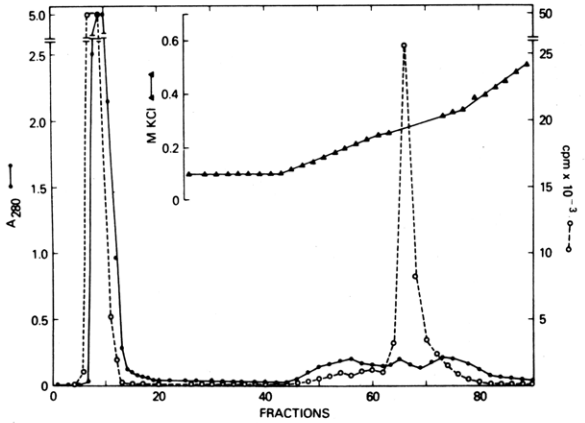

FIGURE 1: Phosphocellulose chromatography. A column (2 cm × 20 cm) of washed cellulose phosphate (Sigma Chemical Co.) was equilibrated with buffer A (see Materials and Methods). A high-speed supernatant (20 mL), containing 140–150 mg of protein, was adsorbed to the column at a flow rate of 4 mL/h. The column was washed with 200–300 mL of buffer A at 8 mL/h and 7-mL fractions were collected. Then a gradient of KCl in buffer A was formed and 3-mL fractions were collected at 8 mL/h. The salt gradient (inset) was determined from conductivity measurements: radioactive methyl incorporation (○); absorbance at 280 nm (●); salt concentration (△).

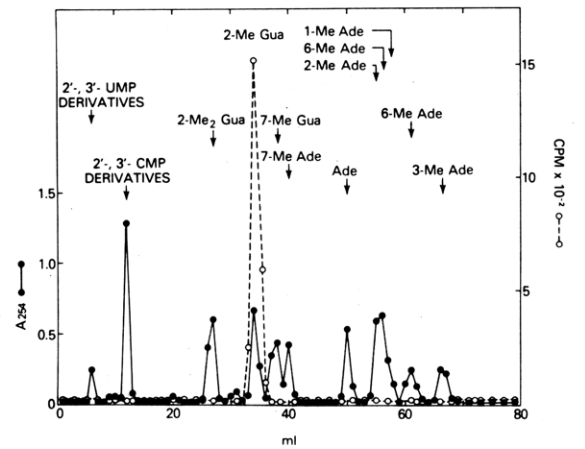

FIGURE 2: Analysis of methylated bases. Between one-half and one-tenth of the total 1 M HCl hydrolysate of a disk from a methylase assay is chromatographed after methylated base standards are added as optical density markers. Total activity is determined by peak summation: absorbance at 254 nm (●); radioactivity incorporated (○); MeGua, methylguanine; MeAde, methyladenine.

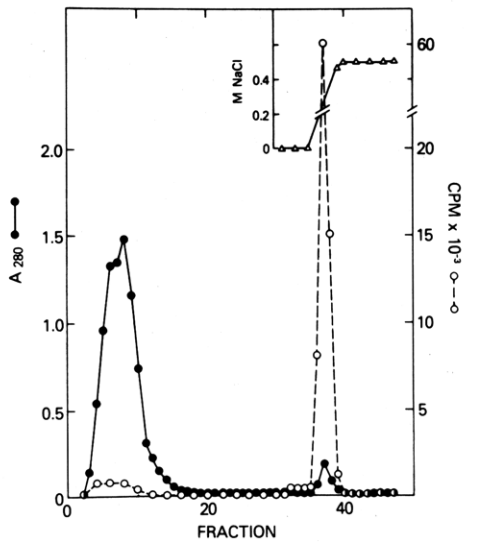

FIGURE 3: Chromatography on SAH–Sepharose. The methylase activity from the phosphocellulose column was precipitated with ammonium sulfate and resuspended in buffer B and adsorbed to the SAH–Sepharose by slow passage (2 mL/h) through the column (0.7 cm × 10 cm). Total protein was 5 mg in 10 mL. The column was washed with 30 mL of buffer B (4 mL/h) and the activity was eluted with 0.5 M NaCl in buffer B: absorbance at 280 nm (●); radioactivity incorporated (○); salt concentration (△).

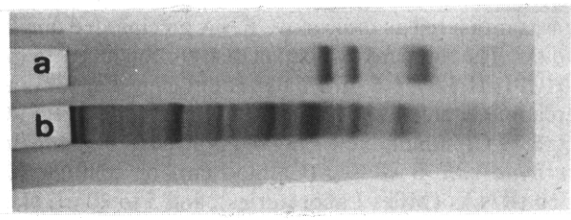

FIGURE 4: Neutral sodium dodecyl sulfate electrophoresis. Sample from SAH–Sepharose chromatography containing 50 μg of protein (a). Sample of the pooled active fractions from phosphocellulose chromatography containing approximately 75 μg of protein (b).

monium sulfate, the enzyme solution was passed through an SAH–Sepharose column, washed with buffer B, and eluted with sodium chloride (Figure 3). A small amount of methylase activity passes through the column along with a large fraction of other proteins. This column gave a 26-fold purification. After both phosphocellulose and SAH–Sepharose chromatography the purification is about 1000-fold and the recovery 5–9%. These calculations are based on the activity in the high-speed supernatant and assume 100% recovery of the N^2-guanine methylase that adsorbs to the phosphocellulose column. However, if the sodium chloride inhibition (see below) of the eluting buffer is taken into account, both the specific activity and percent recovery would be increased several-fold. Efforts to remove the sodium chloride by dialysis and gel filtration resulted in a total loss of activity.

Purity of the protein was examined with acrylamide gel electrophoresis. Numerous bands were seen after the enzyme was chromatographed on phosphocellulose and electrophoresed on either 7.5% acrylamide (Davis, 1964) or 5% sodium dodecyl sulfate–acrylamide gels. Electrophoretic analysis after chromatography on SAH–Sepharose resulted in no detectable protein in the running gel of the 7.5% (or 5%) gel without sodium dodecyl sulfate but a substantial protein band remained at the top of the gel. Analysis for enzyme activity showed that the only region of the gel which contained activity was the top of the running gel where 10–35% of it could be recovered. Sodium dodecyl sulfate–acrylamide gel electrophoresis of the SAH–Sepharose purified enzyme resulted in four bands (Figure 4). It is not known if these bands are impurities or subunits of the enzyme.

Characterization. N^2-Guanine RNA methyltransferase was stable for at least 6 weeks in 10% glycerol, shell frozen and stored at −20 °C. The molecular weight was estimated (Figure 5) to be 77 000 by gel filtration. Magnesium ion was not required for activity, but it stimulated the rate of methylation 1.5-fold with an optimum at 12 mM (Figure 6A). The effects of some other ions are as follows. Ammonium sulfate did not affect the activity up to a concentration of 20 mM; concentrations of 50, 83, 250, and 500 mM resulted in an inhibition of 21, 53, 88, and 95%, respectively. Sodium chloride at concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 M inhibited the activity 40, 76, 90, 97, and 98%, respectively. Potassium chloride inhibition was similar. Sodium phosphate at 8 and 160 mM, pH 8.5, inhibited the activity 26 and 68%, respectively.

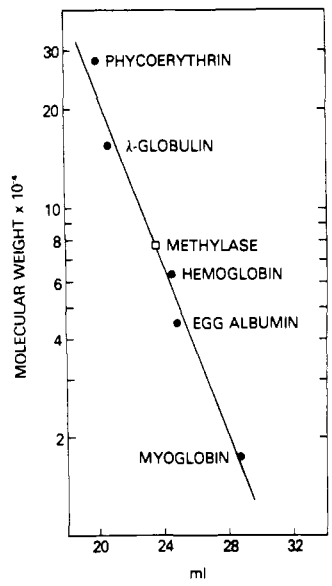

FIGURE 5: Molecular weight determination by gel filtration. One-milliliter samples containing partially purified enzyme or marker proteins (2 mg/mL) in buffer B containing 0.2 M NaCl were applied to a Sephadex G-200 column (1 cm × 40 cm) equilibrated with the same buffer. The samples were eluted at a flow rate of 2 mL/h and 0.4-mL fractions were collected and assayed.

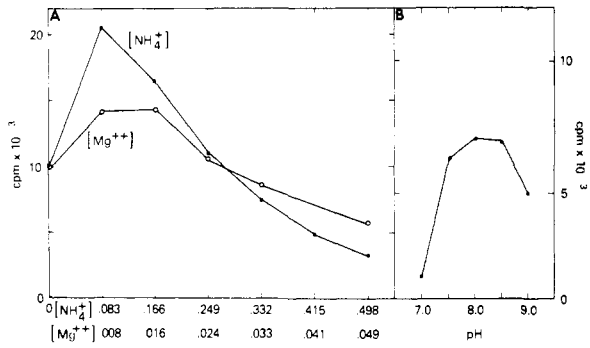

FIGURE 6: (A) Effect of ammonium and magnesium ion concentration on methylase activity. (B) Effect of pH on methylase activity.

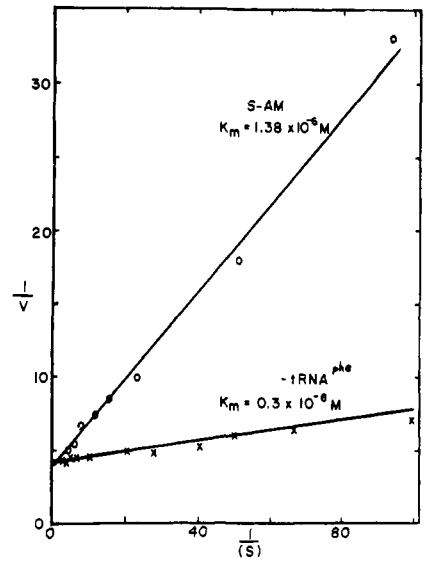

FIGURE 7: Lineweaver-Burk plot varying the concentration of *S*-adenosylmethionine (O) or purified $tRNA^{Phe}$ (X). The K_m for *S*-adenosylmethionine was determined by using mixed *E. coli* tRNA as the methylase acceptor.

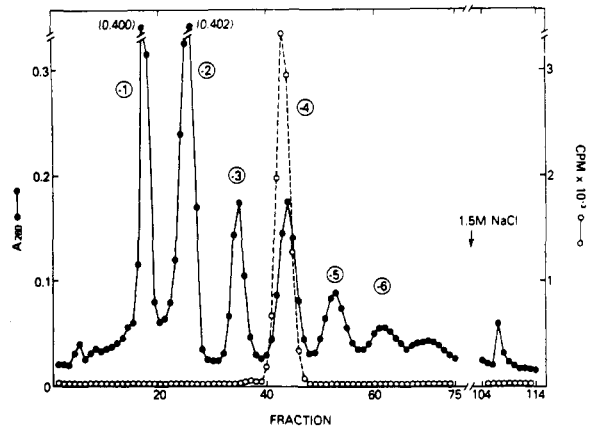

FIGURE 8: Chromatography of a pancreatic RNase digest of methylated $tRNA^{Phe}$. A DEAE-Sephadex A25 column (0.9 cm × 26 cm) was equilibrated with 5 mM Tris-HCl in 7 M urea (pH 7.6) (Rushizky et al., 1964). The methylated $tRNA^{Phe}$ was isolated from a scaled up assay incubation by addition of carrier tRNA (0.5 mg), precipitation with cold ethanol (66%), resuspension in 1 mL of buffer, and two more ethanol precipitations. The final precipitate was suspended in 1.0 mL of 5 mM Tris-HCl, 0.01 M magnesium chloride (pH 7.6), clarified by centrifugation if necessary, and chromatographed on an RPC-5 reversed phase column (Pearson et al., 1971). The radioactive $tRNA^{Phe}$ peak was pooled and 2 mg of carrier tRNA was added and precipitated with cold ethanol. The precipitate was then suspended in 0.6 mL of 0.01 M Tris-HCl (pH 7.8) and digested with 20 μg of pancreatic RNase. An aliquot of the nuclease digestion was then diluted with 4 vol of 0.05 M Tris-HCl in 7 M urea (pH 7.6) and applied to the DEAE-Sephadex A25 column after adding cyclic GMP (−1) and mixed 2′- and 3′-GMP (−2) as optical density markers for the −1 and −2 positions. The column was developed with a salt gradient generated by a closed mixing flask (130 mL) and 0.35 M NaCl in the equilibrating buffer: radioactivity (O); A_{260} (●).

The pH optimum, Figure 6B, is rather broad and peaks at about pH 8.0 to 8.5.

Several commercially purified *E. coli* B amino acid specific tRNAs were tested and found to be substrates. These included $tRNA^{Arg}$, $tRNA^{Leu}$, $tRNA^{fMet}$, $tRNA^{Val}$, and $tRNA^{Phe}$. The best methyl acceptor was $tRNA^{Phe}$, and, from the data shown in Figure 7, the K_m values were determined to be 0.3×10^{-6} and 1.38×10^{-6} M for *S*-adenosylmethionine (with saturating conditions of mixed *E. coli* B tRNA). RNA isolated from chick embryo, chicken kidney, and chicken liver were very poor substrates under the same conditions (less than 10% the acceptor activity of mixed *E. coli* B tRNA).

Nucleotide Position Methylated in E. coli $tRNA^{Phe}$. Since the nucleotide sequence of *E. coli* $tRNA^{Phe}$ is known (Barrell and Sanger, 1969) the exact position methylated could be determined by appropriate enzyme digestions and chromatographic separations. Chromatography of a complete pancreatic RNase digestion of the methylated $tRNA^{Phe}$ resulted in all the radioactivity in the minus four peak (Figure 8) which contains the trinucleotides produced. All five of the expected trinucleotides contain guanine so a T_1 RNase digestion would reduce the possible trinucleotide sequences that contain the N^2-methylguanine to three or two positions depending on the digestion product. Figure 9 shows the DEAE-Sephadex A-25 chromatogram of complete T_1 digestion of the trinucleotide and most of the radioactivity appears in the minus three peak which contains dinucleotides and some appears in the mononucleotide peak. If the digestion time is increased more radioactivity appears in the mononucleotide. This is probably due to a slow splitting of the ApGp dinucleotide which T_1 RNase is known to do (Warrington, 1974). Evidence for this is that after digestion with bacterial alkaline phosphatase and hy-

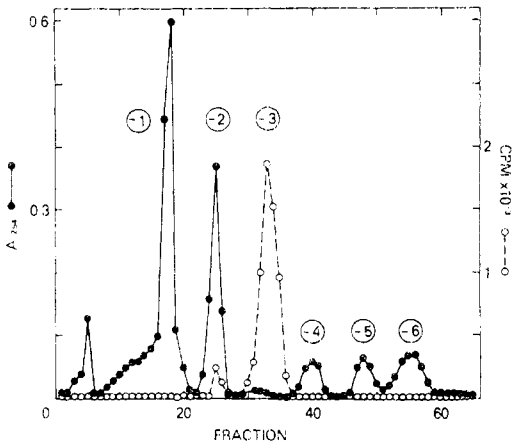

FIGURE 9: Chromatography of T_1 RNase digested trinucleotide peak of pancreatic digest of methylated $tRNA^{Phe}$. An aliquot (1 mL) from the radioactive trimer peak was diluted (1:1) with water and T_1 RNase added (Warrington, 1974). After digestion the sample was then diluted 1:5 with 5 mM Tris-HCl, 7 M urea buffer (pH 7.6) and chromatographed as in Figure 8. The markers added are: cyclic GMP (−1); mixed 2'- and 3'-GMP (−2); $(Ap)_4A$ (−4); $(Ap)_5A$ (−5); $(Ap)_6A$ (−6); radioactivity (○); A_{254} (●).

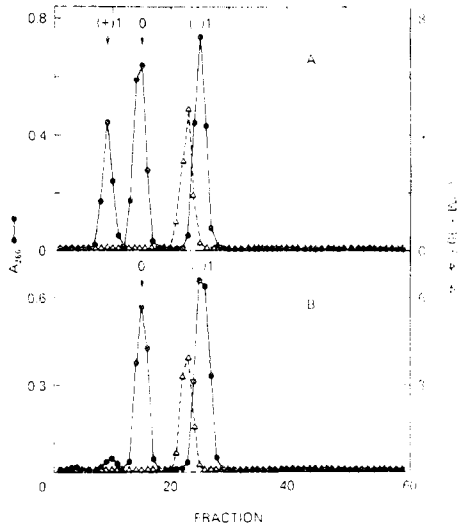

FIGURE 10: Charge comparison at pH 2.7 of radioactive oligonucleotides before and after T_1 RNase digestion of the radioactive trinucleotide generated by pancreatic RNase digestion of methylated $tRNA^{Phe}$. The oligonucleotides were applied to a DEAE-Sephadex A25 column (0.9 cm × 63 cm) equilibrated with 7 M urea-HCl (pH 2.7) (Rushizky et al., 1964). A salt elution gradient was generated as in Figure 8 with 0.2 M NaCl: (A) trinucleotide from pancreatic RNase digest (see Figure 8 for details) plus added adenosine (+1), cyclic AMP (0 charge) and 5'-AMP (−1); (B) dinucleotide from T_1 RNase digest of trinucleotide (see Figure 8 for details) plus added cyclic AMP (0 charge) and 5'-AMP (−1); radioactivity (△); A_{260} (●).

drolysis overnight in 0.2 M potassium hydroxide at 37 °C the radioactivity chromatographed as a nucleoside on DEAE-Sephadex A-25. When the same alkaline phosphatase digestion experiment is performed on the radioactive trinucleotide generated by pancreatic RNase the radioactivity chromatographs as a mononucleotide. This demonstrates that the methylated guanine is the middle base of the trinucleotide. These results narrow the possibilities to two positions, G_{10} (sequence ApGpCp) and G_{15} (sequence ApGphUp). These two trinucleotides can be distinguished from each other by comparison of the charge at pH 2.7 by DEAE-Sephadex chromatography (Rushizky et al., 1964) before and after T_1 RNase digestion. At pH 2.7 both ApGpCp and ApGp resulting from T_1 digestion will have the same −1 net charge and elute at the same position. However, if the sequence ApGphUp, net charge −2, is digested with T_1 RNase, then a loss of one negative charge will result to give a −1 charge which will change the elution position. Figure 10 shows that the elution position is the same before and after T_1 digestion so the trinucleotide and dinucleotide have the same charge which means the sequence must be ApGpCp. Therefore, the G_{10} position in *E. coli* $tRNA^{Phe}$ is the nucleotide that is methylated.

Discussion

A simple two-step procedure has been developed which purifies an N^2-guanine RNA methyltransferase about 1000-fold. The first step, phosphocellulose chromatography, separates the N^2-guanine methylase from the other methylases, and the second step, adsorption to an SAH-Sepharose column and elution with salt, provides the specificity of affinity chromatography. Affinity chromatography was first used by Taya and Nishimura (1973) to purify an RNA methylase. They conjugated methyl-deficient $tRNA^{Glu}$ to Sepharose 4B and eluted the adsorbed methylase with potassium chloride as the final purification step. They also used *S*-adenosylhomocysteine as the ligand bound to Sepharose to purify the same methylase (Taya and Nishimura, 1974) and suggested this ligand would be generally valuable for other RNA methylases. Though there are exceptions (Glick et al., 1975), SAH is particularly promising because it seems to generally bind to RNA methylases and is considerably more stable than *S*-adenosylmethionine.

It is not certain that the SAH-Sepharose column separates the methylase from the other proteins on the basis of specific affinity for SAH rather than by more general adsorption phenomena. The fact that all the enzyme activity and protein pass through the column when glycine is substituted for SAH suggests the binding is specific. Experiments where the column was eluted with SAH resulted in inability to detect the activity. The activity was not recovered after passage of elution fractions through Sephadex G-25 or dialysis. Presumably the methylase was eluted because subsequent salt elution of the column did not release any activity. These experiments are complicated by the fact that at this stage of purity activity is easily lost and SAH at low concentrations is a potent inhibitor.

Many attempts were made to band this enzyme in an acrylamide gel system so that the activity could be associated with a stained protein band. Curiously, this highly purified enzyme barely entered 7.5 or 5% acrylamide gels even though its molecular weight is well below the pore limits of the gels. As much as 35% of the enzyme activity could be recovered at the top of the running gel. Included in the attempts to find conditions under which it would enter the gel are: electrophoresis at different pHs; presence of 1% Triton X-100 or thioglycolate; digestion of the protein sample with lipase, RNase, or DNase; photopolymerization in a sample gel.

The general properties of this enzyme are similar to the few other methylases examined (Kuchino and Nishimura, 1970; Agris et al., 1974; Kraus and Staehelin, 1974a,b; Glick et al., 1975). For example, it becomes more unstable and difficult to handle as purity increases. Its molecular weight, K_m values for tRNA and *S*-adenosylmethione, response to ammonium ions, and pH optimum vary only marginally from the other enzymes as a group, and it methylates a position (G_{10}) which is commonly found in eukaryote RNAs.

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Peroxidase-Catalyzed Oxidation of Protein Sulfhydryls Mediated by Iodine†

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ABSTRACT: Lactoperoxidase, myeloperoxidase, or horseradish peroxidase catalyzed the peroxide-dependent oxidation of protein sulfhydryls in the presence of iodide. Sulfhydryl oxidation was mediated by the oxidation of iodide to iodine. About 1 mol of sulfhydryls of bovine serum albumin or β-lactoglobulin was oxidized per mol of peroxide or iodine. Sulfhydryls were oxidized to the sulfenyl iodide derivative as indicated by the loss of iodide from solution and formation of a derivative that reacted with the sulfenyl-specific reagent, 4,4′-bis(dimethylamino)thiobenzophenone (thio-Michler's ketone). Peroxidase-catalyzed oxidation of sulfhydryls was proportional to peroxide and independent of iodide concentration over a wide range. Therefore, each iodide ion could participate in the oxidation of a number of sulfhydryls. At low iodide concentrations, the yield of sulfenyl derivatives also exceeded the amount of iodide. These results indicated that sulfenyl iodide was in equilibrium with sulfenic acid and free iodide ion. At low iodide concentrations, release of iodide from sulfenyl iodide permitted reoxidation of iodide to iodine. When the initial sodium iodide concentration was less than 1 μM, no sulfhydryl oxidation was detected regardless of peroxide or peroxidase concentrations. Also, over a narrow range of iodide concentrations, sulfhydryl oxidation was not proportional to peroxide. Under these conditions, depletion of iodide and competition by electron donors other than iodide appeared to limit iodine formation. Although a lower limit for iodide turnover was observed, only small amounts of iodide were required for oxidation of large amounts of protein sulfhydryls. Therefore, in comparing the effects of iodine with those of the peroxidase system, the valid quantitative comparison can be between iodine and peroxide concentrations, rather than between iodine and iodide concentrations.

Peroxidase-catalyzed iodination has a role in resistance to viral and microbial infection (Klebanoff, 1975), and in thyroid hormone synthesis (Morrison, 1973). Also lactoperoxidase-catalyzed iodination has been used to introduce radioactive label into proteins (Morrison et al., 1971a) and as a vectorial probe of biological membrane structure (Phillips and Morrison, 1970, 1971; Morrison et al., 1974) and protein structure (Morrison and Schonbaum, 1976).

Peroxidases can oxidize iodide (I^-) to iodine (I_2), which in turn can iodinate biological components. However, lactoperoxidase catalyzes iodination of accessible tyrosine residues by a mechanism that does not involve a diffusible intermediate such as I_2 (Morrison and Bayse, 1970; Morrison and Schonbaum, 1976). It has been suggested that peroxidase-catalyzed antimicrobial and antiviral activity is not due to I_2 (Klebanoff, 1967, 1975), and that iodination of thyroglobulin may involve protein-bound iodinating agents (Maloof and Soodak, 1963; Jirousek and Cunningham, 1968).

Sulfhydryls of BSA[1] and β-lactoglobulin are located in clefts

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[1] Abbreviations used: BSA, bovine serum albumin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid; TMK, 4,4′-bis(dimethylamino)thiobenzophenone (thio-Michler's ketone); $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.