EVIDENCE FOR THE ENZYMATIC METHYLATION OF CRYSTALLINE OVALBUMIN PREPARATIONS

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When L-phenylalanine-U-C^{14} was used in this laboratory as a standard to test for amino acid incorporation into cell-free extracts of rat skin, it was found to require ATP, a 105,000xg cell supernatant fraction and RNP particles. Incorporation was enhanced by GTP and it was inhibited by RNase and puromycin. Thus phenylalanine incorporation was found to be consistent with other amino acid incorporating systems that are known, (Fruton, 1963). However, when L-methionine-$^{14}CH_3$ was used, it was found that radioactive labeling of the protein fraction required only the cell supernatant fraction and ATP. It did not require RNP particles, it was not enhanced by GTP and it was not inhibited by puromycin or RNase. The results were consistent with an enzymatic methylation reaction since it was also found that ATP and L-methionine-$^{14}CH_3$ (L-met-$^{14}CH_3$) could be replaced by S-adenosylmethionine-$^{14}CH_3$ (SAM-$^{14}CH_3$),(Edelstein and Liss, 1966). Although the number and types of biological compounds which can be methylated by S-adenosylmethionine is extensive, the enzymatic methylation of a protein has not previously been described. The results obtained in this laboratory now indicate that S-adenosylmethionine is also capable of methylating protein enzymatically.

EXPERIMENTAL AND RESULTS

The methylating enzyme activity could be detected in 0.05M sodium bicarbonate dialyzed, 198,000xg cell supernatant fractions of rat skin and spleen, rat appendage tumor and mouse epithelial tumor without the addition of any exogenous protein. However, in partially purified enzyme preparations containing minimal endogenous activity, commercial preparations of 5-times crystallized ovalbumin (OA) could serve as a suitable acceptor (Tables 1, 2; Fig. 1b). The ovalbumin was a product of Mann Research Laboratories, Inc.

To prepare partially purified methylating enzyme, minced rat skin was homogenized in 5 volumes of 0.02M Tris-HCl buffer, pH 7.5, containing 3mM 2-mercaptoethanol. The homogenate was centrifuged first at 20,000xg at 4° and the supernatant solution recentrifuged for 60 minutes at 198,000xg. The solution was dialyzed overnight against 0.05M $NaHCO_3$ and then applied to a 2 x 30 cm DEAE-cellulose column (Fig. 1a). In the case of rat skin, the methylating activity was found in peak B and it was concentrated by precipitation at 0 - 90% ammonium sulfate saturation. The sediment was disolved in 0.02M Tris-HCl buffer, pH 7.5, containing 3mM mercaptoethanol and dialyzed against the same buffer. The methylating activity in extracts from rat appendage tumor, rat spleen and mouse epithelial tumor were all prepared in the same manner with the exception that in these tissues the methylating activity was detected, using similar DEAE-cellulose columns, in the peak A area and it could be precipitated by 0 - 60% ammonium sulfate fractionation. No methionine-activating enzyme activity could be detected in this fraction (Fig. 1a).

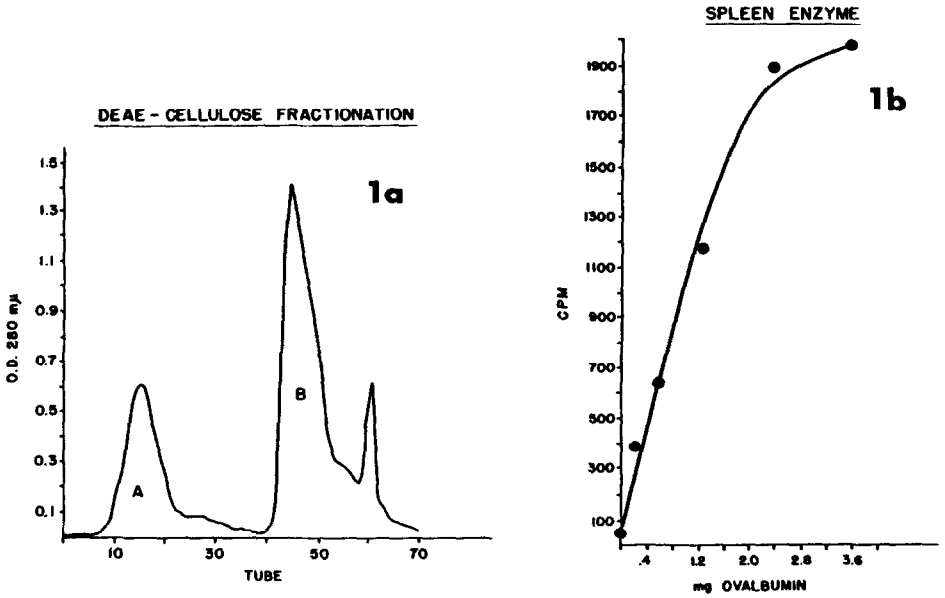

Fig. 1a. DEAE-cellulose elution pattern for purification of methylating enzyme from rat skin. Peak A was eluted by equilibrating solution, 0.05M $NaHCO_3$. Subsequent elution of column was done by using a gradient of 0-0.5M NaCl in 0.05M $NaHCO_3$. Peak B from rat skin preparations contained both methionine-activating enzyme and methylating enzyme. Purification of methylating enzyme from rat spleen and appendage tumor and mouse epithelial tumor gave a similar type of elution profile, but the methylating enzyme washed through with equilibrating 0.05M $NaHCO_3$ solution and was found in a peak located similarly to peak A.

Fig. 1b. Incorporation of C^{14} into the trichloroacetic acid insoluble protein fraction in the presence of SAM-$^{14}CH_3$ and purified rat spleen enzyme (0-60% ammonium sulfate fraction). Assays were carried out as described in Table 2.

Methylating enzyme preparations from all tissues could be stored frozen in 0.02M Tris-HCl buffer, pH 7.5, containing 3mM 2-mercaptoethanol for 4-6 weeks without any significant loss of activity. Since the peak containing the methylating enzyme in skin extracts (peak B) was found also to contain methionine-activating enzyme (ATP:L-methionine S-adenosyl transferase) which

TABLE 1

Activity of Methionine-Activating and Methylating Enzymes in Purified Extracts of Rat Skin

Ovalbumin added	SAM generating system	Incorporation into protein	Incorporation into SAM
mg		cpm	
0	L-Methionine-$^{14}CH_3$ + Mg^{++}	---	0
0	L-Methionine-$^{14}CH_3$ + ATP + Mg^{++}	---	1,700
0	DL-Methionine-$^{14}COOH$ + Mg^{++}	---	0
0	DL-Methionine-$^{14}COOH$ + ATP + Mg^{++}	---	3,300
1.2	L-Methionine-$^{14}CH_3$ + ATP + Mg^{++}	403	---
1.2	L-Methionine-$^{14}CH_3$ + ATP	23	---
0	L-Methionine-$^{14}CH_3$ + ATP + Mg^{++}	88	---
1.2	DL-Methionine-$^{14}COOH$ + ATP + Mg^{++}	17	---
1.2	DL-Methionine-$^{14}COOH$ + ATP	15	---

For assay of methionine-activating activity the incubation mixture in 0.02M Tris-HCl buffer, pH 7.5, with 3mM 2-mercaptoethanol contained skin protein 0.8mg (0-90% ammonium sulfate fraction of peak B); $MgCl_2$, 3.5nmoles; ATP, 3µmoles; L-methionine-$^{14}CH_3$, 2.5nmoles (10,000cpm) or DL-methionine-$^{14}COOH$, 25nmoles (40,000 cpm) in a final volume of 0.22 ml. The samples were incubated for 60 minutes at 35° and assayed for SAM synthesis using Dowex 50-X4 (NH_4^+) columns according to the procedure of Mudd et al.(1965). To assay for protein methylating activity, similar samples containing reactants in the same concentration were incubated for 60 minutes at 35°. The reactions were stopped by the addition of 0.1 ml of 15% trichloroacetic acid, the precipitate was washed 3 times with 5% trichloroacetic acid and the sediments collected on millipore filters. Ovalbumin was routinely added as a control to all samples which had been incubated without ovalbumin immediately before the addition of 15% trichloroacetic acid.

had a Mg^{++} requirement (Table 1), the C^{14} labeling of the protein fraction in the presence of ovalbumin was therefore possible using SAM-$^{14}CH_3$ or an SAM-$^{14}CH_3$ generating system consisting of L-methionine-$^{14}CH_3$, ATP and Mg^{++}. The methylation reaction employing SAM-$^{14}CH_3$ was not enhanced by the addition of Mg^{++}. No labeling of the protein fraction occurred with DL-methionine-$^{14}COOH$. It can be concluded, therefore, that the reaction is indeed a methylation reaction and not a nonenzymatic incorporation of methionine residues after the enzymatic formation of methionyladenylate, (Castelfranco, et al. 1958). In addition, no radioactive methionine could be detected in the N-terminal position by analysis using 1, 2, 4-fluorodinitrobenzene (FDNB). The incorporation into the protein fraction was found to be dependent on the concentration of ovalbumin (Fig. 1b), to increase with time and it was proportional to enzyme concentration within the experimental limits of the assay system (Table 2).

Mudd (1962, 1963) has demonstrated that although SAM is reversibly bound by methionine-activating enzyme of bakers' yeast, it is removed by treatment with cold dilute trichloroacetic acid. We are not dealing with an analogous type of binding since the counts cannot be removed by trichloroacetic acid. Further, Mudd has reported that SAM is not bound by ovalbumin, an observation we have confirmed.

The evidence which indicates that the enzyme is indeed methylating the protein fraction is based upon the findings that the radioactivity is not extractable with hot trichloroacetic acid, nor organic solvents (alcohol, ether, chloroform, benzene), nor was the activity diminished by preincubation with large amounts of crystalline pancreatic ribonuclease. The radioactivity also accompanied ovalbumin on separation of the high and low molecular

TABLE 2

Enzymatic Methylation of Ovalbumin

Enzyme source	Enzyme	OA	Incubation	Incorporation into protein		
	mg	mg	minutes	+OA	cpm	-OA
Mouse epithelial tumor						
Dialyzed 198,000xg supernatant	0.5	2.4	60	480		341
0-60% ammonium sulfate fraction	0.2	2.4	60	2366		127
Rat appendage tumor						
0-60% ammonium sulfate fraction	0.6	2.4	60	1722		150
Rat Spleen						
0-60% ammonium sulfate fraction	0.24	2.4	60	2337		44
"	0.08	2.4	60	994		---
" (heated 100°,1 min.)	0.24	2.4	60	14		---
"	0.24	2.4*	60	760		---

Assays for methylating activity were carried out in incubation mixtures composed of SAM-$^{14}CH_3$, 2nmoles (40,000 cpm); ovalbumin (OA) and enzyme as indicated in a final volume of 0.2 ml. The reactions were stopped by the addition of 0.1 ml of 15% trichloroacetic acid, washed with 5% trichloroacetic acid and collected in millipore filters. Ovalbumin was routinely added to all samples which were incubated without ovalbumin immediately before the addition of 15% trichloroacetic acid. Zero time controls were all negative.

*Ovalbumin solution was preheated in boiling water for 2 minutes before addition of other reactants and incubation.

weight components of the incubated mixture on Sephadex G-25 columns. The 5x-crystallized ovalbumin gave several neighboring bands on starch gel electrophoresis a result which is consistent with the known multicomponent nature of the protein (Gottschalk and Graham, 1966). In order to determine if there might be in the ovalbumin preparations a trace contaminant of low molecular weight which was being methylated, the ovalbumin was purified by DEAE-cellulose column chromatography, recrystallized by means of ammonium sulfate, dialyzed and lyophilized. Such repurified preparations gave several neighboring bands on starch gel electrophoresis and served just as well as an acceptor in the enzymatic reaction. Further, aliquots of an ovalbumin solution were heated by immersion in a boiling water bath for 2 minutes, and then SAM-$^{14}CH_3$ and spleen enzyme were added to the heat coagulated ovalbumin sample after cooling. After incubation for 60 minutes at 35°, it was found that the incorporation of C^{14} into the protein fraction was substantially less than in the incubations which were carried out using unheated ovalbumin (Table 2).

Various protein acceptors have been tested in the reaction, but until now the use of crystalline ovalbumin and unfractionated sericin, the soluble protein of silk, have resulted in the greatest incorporation. Human albumin, bovine fibrinogen and various gamma globulin preparations in equivalent concentrations gave only approximately one-fourth to one-third of the activity as compared with ovalbumin.

The reaction product has not yet been identified. However, it has been found that the radioactivity in the trichloroacetic acid protein sediment can be practically quantitatively recovered as a volatile component after hydrolysis for 2 hours at 110° using 3N NaOH in sealed tubes. A vacuum pump and an alcohol-dry ice

trap are used to collect the distillate. After alkaline hydrolysis, the radioactivity remains volatile if the sample remains alkaline or is first acidified before distillation. This suggests that the volatile product is probably not an acid or a base. On the assumption that the product is a methylated amino acid residue in the protein, the radioactive protein fraction was hydrolyzed for 20 hours at 110° in vacuum-sealed tubes using 6N HCl. Under such conditions, the radioactivity can also be detected as a volatile component in the course of removing the HCl under vacuum. It is known that ε-N-methyllysine is present in some proteins, (Murray, 1964; Ambler and Rees, 1959). However, the formation of ε-N-methyllysine residues can be excluded since it is known to be relatively stable to alkaline hydrolysis, (Neuberger and Sanger, 1944). Therefore, it must be some other amino acid residue in the ovalbumin molecule which is being methylated.

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