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S-Adenosylmethionine:Protein Methyltransferases in Hepatomas[1]

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SUMMARY

Protein methylase III (*S*-adenosylmethionine:protein-lysine methyltransferase; EC 2.1.1.25) and protein methylase I (*S*-adenosylmethionine:protein-arginine methyltransferase; EC 2.1.1.23) activities were examined in isolated nuclei and cytosol fraction, respectively, from various hepatomas with different growth rates. The enzyme activities of both enzymes paralleled the rates of tumor growth in fast- and moderately growing hepatomas. The parallelism was more evident with protein methylase I than with protein methylase III. While protein methylase III activity was elevated in the fast- to moderately growing hepatomas, the enzyme that is responsible for demethylating proteins, ε-alkyllysinase (ε-alkyl-L-lysine:oxygen oxidoreductase; EC 1.5.3.4), had an inverse relationship to the rate of tumor growth, thus suggesting a possible physiological antagonism. When isolated rat liver nuclei were methylated *in vitro* with *S*-adenosyl-L-[*methyl*-^{14}C]methionine as methyl donor, H_2SO_4-insoluble protein and histones had almost equal amounts of *methyl*-^{14}C incorporated. However, amino acid analysis revealed that methylated arginines are the predominant form of radioactivity in the H_2SO_4-insoluble protein (product of protein methylase I), while methylated lysines are the major methylated amino acids in the histones (product of protein methylase III). Furthermore, the hydrolysate of the H_2SO_4-insoluble protein showed four unknown radioactivity peaks on the amino acid analyzer in addition to the known methylated arginine and lysine derivatives.

INTRODUCTION

It is now well recognized that basic and acidic amino acid residues of some proteins are methylated *in vivo* (3, 9, 15, 19). The methylation of the side chains occurs subsequent to the synthesis of the polypeptide bonds (5). During the past several years, various amino acid-specific protein methyl-transferases have been identified. Protein methylase I (*S*-adenosylmethionine:protein-arginine methyltransferase; ED 2.1.1.23) methylates the guanidino group of arginine residues, protein methylase II (*S*-adenosylmethionine: protein-carboxyl methyltransferase; EC 2.1.1.24) methylates free carboxyl group of aspartic and glutamic acid residues, and protein methylase III (*S*-adenosylmethionine: protein-lysine methyltransferase; EC 2.1.1.25) methylates the ε-amino group of lysine residues. We have previously observed that protein methylase I activity in various hepatomas roughly paralleled the growth rate of the hepatomas and that protein methylase II and III activities did not change significantly (20).

In a continued effort to elucidate the biochemical significance of protein methylation in general and protein methylases in tumors in particular, we reexamined the protein methylase I and III activities in various tumors under slightly different assay conditions. Since there exists an enzyme ε-alkyllysinase (ε-alkyl-L-lysine:oxygen oxidoreductase; EC 1.5.3.4) which demethylates [*methyl*-^{14}C]histone (4, 16, 18), the ε-alkyllysinase activity in various tumors was also determined in ralation to protein methylase III.

MATERIALS AND METHODS

Materials. *S*-Adenosyl-L-[*methyl*-^{14}C]methionine (specific activity, 28.9 Ci/mole in an aqueous solution, pH 3.0) was purchased from Amersham:Searle Corp., Arlington Heights, Ill., and [^{14}C]formaldehyde (specific activity, 59.0 Ci/mole in an aqueous solution) from New England Nuclear, Boston, Mass. Phenazine methosulfate and histone type 2A (a mixture of various histone fractions isolated from calf thymus) were obtained from Sigma Chemical Co., St. Louis, Mo.; 5,5-dimethyl-1,3-cyclohexanedione (Dimedon) from Eastman Kodak Co., Rochester, N. Y.; and semicarbazide hydrochloride from Fisher Chemical Co., King of Prussia, Pa. All remaining chemicals were obtained from either Sigma or Fisher.

Tumor-bearing Animals. The Morris hepatomas used in this study were carred in male Buffalo rats (by bilateral transplantation in the femoral muscle) at Howard University and then shipped periodically to Philadelphia. The Novikoff hepatomas were carried in Sprague-Dawley rats (obtained from Charles River Breeding Laboratories, Wilmington, Mass.) induced by bilateral i.m. or s.c. transplantation, or i.p., in which case they grew as free cells in the ascites fluid. Animals were fed *ad libitum* on Purina rat chow and all were housed at a constant temperature of 25° with 12 hr of light daily, automatically controlled. The general properties of the Morris and Novikoff hepatomas used in this study are described in Table 1.

Enzymatic Assays. The animals were killed by decapitation at about the same time in the morning to avoid possible diurnal effects on the enzymes. The protein methylase I and III activities were assayed according to the method de-

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scribed earlier (20). However, the protein methylase I activity was determined with cytosol fraction (105,000 × *g* supernatant of whole homogenate homogenized in 0.25 M sucrose containing 3×10^{-3} M $CaCl_2$) and the protein methylase III activity was determined with the 700 × *g* precipitate. Since protein methylase I is mostly localized in the cytosol fraction (13) and protein methylase III in the nucleus (14), this prior separation for the enzymatic assay accentuated the changes in the enzyme activity (compare Charts 1 and 2 in this paper with those in Ref. 20).

ε-Alkyllysinase activity in liver and kidney (Table 2) was determined by the method described previously (18) using ε-*N*-mono[*methyl*-^{14}C]-L-lysine as substrate, and the enzyme activity was expressed as cpm liberated as formaldehyde per hr per mg of enzyme protein. Protein concentration was estimated by the method of Lowry *et al.* (8) using bovine serum albumin as standard.

***In Vitro* Methylation of Isolated Rat Liver Nuclei.** Twenty-one g of rat liver were homogenized in 0.25 M sucrose containing 3×10^{-3} M $CaCl_2$ by an electrically driven Teflon-glass homogenizer. The sucrose solution was 9 times the volume of the rat liver. The homogenate was passed through a double layer of cheesecloth and was centrifuged at 700 × *g* for 10 min. The pellet was washed twice with the above sucrose solution and was resuspended in 2.4 M sucrose. The suspension was centrifuged in a swinging-bucket SW-25 rotor at 63,600 × *g* (25,000 rpm) for 60 min (22). The isolated nuclei were suspended in 2.3 ml of 0.25 M sucrose containing 3×10^{-3} M $CaCl_2$. This nuclear suspension was incubated with 1.0 ml of *S*-adenosyl-L-[*methyl*-^{14}C]methionine (0.05 mmole; 4.18×10^6 cpm) and 1.7 ml of 0.5 M phosphate buffer, pH 7.2, at 37° for 2 hr. Various protein fractions (Table 3) were prepared by adding 0.6 ml of 2 N H_2SO_4 to the above incubation mixture and centrifuging the mixture at 10,000 × *g* for 10 min (12). The resulting precipitate was washed twice with 0.25 N H_2SO_4 to yield an H_2SO_4-insoluble fraction. To the combined supernatant and wash 2.5 volumes of 95% cold ethanol were added and the suspension was left overnight at −10°. The solution was centrifuged and the precipitate was further washed 3 times with 95% cold ethanol to give an ethanol-soluble fraction. The precipitate was suspended in 10 ml of water and was further extracted 3 times with water to yield the sulfated histones. The remaining residual protein is designated as H_2O-insoluble fraction.

In order to investigate the distribution pattern of radioactivity in the above protein fractions, the individual protein fractions were hydrolyzed in 6 N HCl under reduced pressure at 110° for 24 hr and the hydrolysates were analyzed by a Perkin-Elmer automatic amino acid analyzer with pH 5.84 buffer system (11), and the radioactivity was monitored by flow cell of Packard Tri-Carb Model 2002 liquid scintillation spectrometer with counting efficiency of approximately 60%.

Table 1

History and biology of Novikoff and various Morris hepatomas

Tumor	Generation	Mos. between transfer	Days of growth	Degree of differentiation
Novikoff		0.5	14	Poor
Morris				
7288Ctc	98	0.5	18	Poor
9618A$_2$	77	1.0	30	Poor
8995	52	2.0	35	Good
5123C	107$_2$	2.0	87	Good
7794A	46	2.8	360	Good
44	12	5.8	154	Good
28A	13	6.0	250	Good
47C	10	7.7	150	Good
9618B	12	10.0	123	Good to high
66	5	11.5	157	High

Table 2

Level of ε-alkyllysinase activity in kidney and liver of rats bearing tumors of various growth rates

The values are the average of 3 to 4 animals. The whole homogenate from each animal was assayed for the enzyme activity with duplicates. The rest of the experimental procedures are described under "Materials and Methods."

Hepatoma	Enzyme activity (cpm/hr/mg of enzyme protein)	
	Kidney	Liver
Novikoff	1904 ± 172[a]	289 ± 61
Morris		
7288	2120 ± 121	502 ± 84
9618A$_2$	2054 ± 234	492 ± 16
8995	1921 ± 171	319 ± 18
5123C	2630	551
7794A	2654 ± 650	465 ± 62
44	2134 ± 155	463 ± 161
28A	2055 ± 131	525 ± 112
47C	2527 ± 323	682 ± 192
9618B	2135 ± 163	572 ± 30
66	3188 ± 160	689 ± 115

[a] Mean ± S.D.

Table 3

Fractionation of the endogenous proteins of isolated rat liver nuclei labeled with S-adenosyl-L-[methyl-^{14}C]methionine

Isolated rat liver nuclei, 2.3 ml; *S*-adenosyl-L-[*methyl*-^{14}C]methionine (0.05 mmol; 84 cpm/pmole), 1.0 ml; and 0.5 M phosphate buffer 1.7 ml at pH 7.2 were incubated at 37° for 2 hr. Fractionation of the endogenous proteins was carried out according to the method previously described (12). A portion of each fraction was treated to remove unreacted *S*-adenosyl-L-[*methyl*-^{14}C]methionine, nucleic acids, and phospholipids, except for the ethanol-soluble fraction which was not treated with ethanol to remove phospholipids. More detailed experimental procedures are described under "Materials and Methods."

Fraction	Protein		Radioactivity		Specific activity (cpm/mg)
	mg	%	cpm	%	
H_2SO_4-insoluble	37.3	56.2	57,050	45.3	1,530
Histones, crude	22.1	33.3	59,790	47.5	2,705
Ethanol-soluble	Trace		1,330	1.0	
H_2O-insoluble	7.0	10.5	7,820	6.2	1,117

RESULTS

General Properties of Tumors. Table 1 lists the history and characteristics of the various tumors used in this study. Tumors are arranged in the order of the average time

between transfer which gives a rough estimation of their rate of growth. This time between transfer is evaluated on the basis of: (*a*) size of the tumor; (*b*) characteristics of the type of tumors; and (*c*) physical condition of the animal. Tumors vary from poorly differentiated tumors, such as Novikoff hepatoma and Morris hepatomas 7288 and 9618A$_2$, to well-differentiated tumors, such as Morris hepatomas 9618B and 66.

Protein Methylase III Activity in Various Hepatomas. Chart 1 illustrates the levels of protein methylase III activity in the isolated crude nuclear fraction of tumors with varying growth rate. Although there is some scattering of the values, the direct proportionality between the enzyme activity and the rate of tumor growth with fast-growing hepatomas is apparent. Due to prior separation of the nuclear fraction for the enzymatic assay, the results shown in Chart 1 are for superior to those published previously for whole homogenate (20).

ε-Alkyllysinase Activity in Various Tumors. We have recently observed that ε-alkyllysinase demethylates not only free ε-*N*-mono[*methyl*-^{14}C]-L-lysine but also enzymatically or nonenzymatically methylated [*methyl*-^{14}C]histone, producing [^{14}C]formaldehyde (18). Furthermore, the activity of protein methylase III (which methylated the ε-amino group of lysine residues) follows a pattern that appears to be antagonistic to the activity of ε-alkyllysinase (which demethylates the methyl group incorporated into the ε-amino group); when protein methylase III activity is high, as in the case of young rat liver or kidney, ε-alkyllysinase activity is low. On the other hand, when the former activity is low, as in the case of old rat liver or kidney, the latter activity is elevated (18). These findings indicate a possible physiological interrelationship between these 2 enzymes, since these 2 reactions appear to oppose each other. Therefore, it was thought to be of interest to examine the kind of relationship that exists between these 2 enzyme activities in tumors of varying growth rates.

Chart 2 illustrates the levels of ε-alkyllysinase activity in the Novikoff and various Morris hepatomas with different rates of growth. It is evident from the chart that the levels of

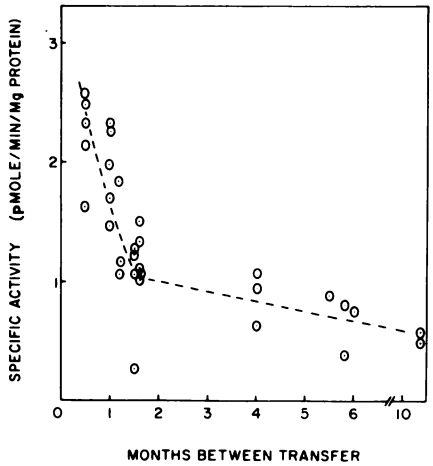

Chart 1. Protein methylase III activity in hepatomas with varying growth rates. The values are the average of 2 animals. Duplicate assays were run with each animal.

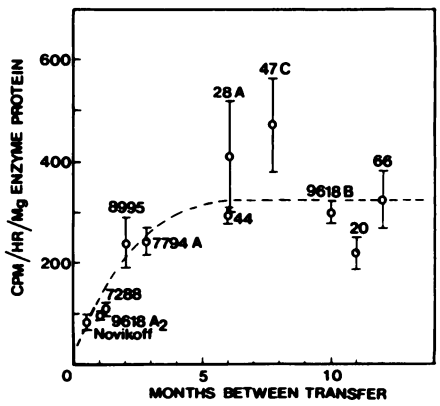

Chart 2. ε-Alkyllysinase activity in hepatomas with varying growth rates. The values are means ± S.D. for 3 or 4 animals. Duplicate assays of tissue whole homogenate were run with each tissue. *Numbers*, Morris hepatoma numbers (history and biological data are described in Table 1); *bars*, S.D.

the enzyme activity exhibit a pattern that is exactly opposite to that depicted for protein methylase III in Chart 1; as the tumor grows faster, ε-alkyllysinase activity is present at low levels. Although ε-alkyllysinase activity in the fast-growing hepatomas is lower, the enzyme activity in the host liver and kidney does not change significantly, except in the host liver of rats bearing the Novikoff hepatoma and Morris hepatoma 8995 (Table 2). In these 2 instances, ε-alkyllysinase activity was significantly lowered. It was found previously that the protein methylase III activity in the host liver of rats bearing the Novikoff hepatoma was significantly elevated (20).

Protein Methylase I Activity in Tumors. Protein methylase I methylates the guanidino group of arginine residues of protein substrate, and the enzyme is most abundant in the cytosol fraction of all the organs tested (19). When protein methylase I activity is measured in the cytosol of various hepatomas with different growth rates, there exists an impressive correlation between the enzyme activity and the rates of tumor growth (Chart 3). Protein methylase I activity in the Novikoff hepatoma is more than 6 times higher than the enzyme activity in Morris hepatoma 20. The parallelism between the enzyme activity and the rate of tumor growth is most evident among the fast-growing hepatomas.

An enzymatic mechanism that demethylates free N^G-methylated arginine or N^G-methylated arginine residues in protein molecules has not yet been identified. Therefore, a relationship such as that of protein methylase III and ε-alkyllysinase activity cannot be examined. However, the potential importance of the protein methylase I reaction is evident not only from the result shown in Chart 3 but also in the observation that the rate of *methyl*-^{14}C incorporation into the guanidino group of arginine residues of H_2SO_4-insoluble rat liver nuclear protein is extremely high (see below). The potential significance of H_2SO_4-insoluble chromatin protein ("acidic protein") has been recently reviewed by Stein and Baserga (21).

In Vitro **Methylation of Isolated Rat Liver Nuclei.** "Pure" nuclei preparation isolated from normal rat liver

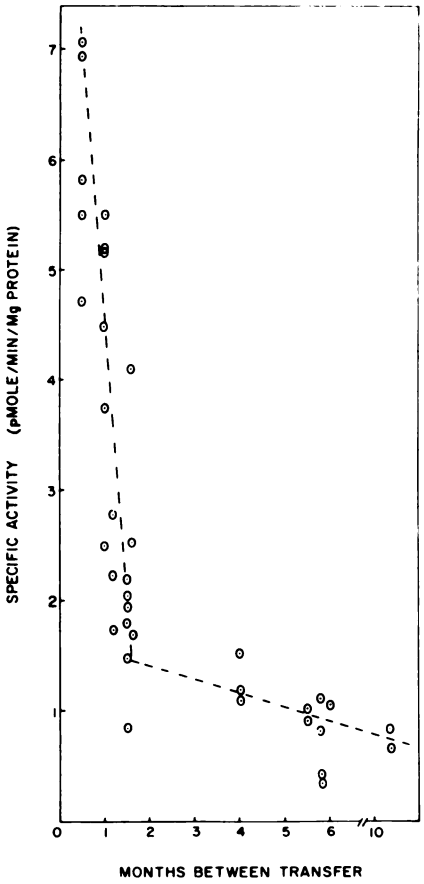

Chart 3. Protein methylase I activity in hepatomas with varying growth rates. The conditions are the same as in Chart 1.

was incubated with *S*-adenosyl-L-[*methyl*-^{14}C]methionine, and various protein fractions were fractionated according to their solubilities. As seen in Table 3, the radioactivity derived from *S*-adenosyl-L-[*methyl*-^{14}C]methionine is distributed equally among the H_2SO_4-insoluble protein (acidic protein) and crude histones. Because only a small amount of protein in the rat liver nuclei exists as histones (Table 3, Column 3), the specific radioactivity in the histone fraction is somewhat higher than that of the H_2SO_4-insoluble protein fraction.

Although the radioactivity is distributed almost equally among the H_2SO_4-insoluble protein and the histones, the patterns of *methyl*-^{14}C incorporated into various amino acid side chains are quite different. As shown in Chart 4, most of the radioactivity in the histones is incorporated into the ε-amino group of lysines, as ε-*N*-mono-, ε-*N*-di-, and ε-*N*-trimethyllysine. On the other hand, the radioactivity incorporated into the H_2SO_4-insoluble protein fraction was found to exist mostly as methylated arginines, as N_G-mono-, N^G,N^G-di-, or N^G,N'^G-dimethylarginine (Chart 5). Furthermore, a large amount of radioactivity was also found in those compounds that have not yet been characterized. Further studies on the identification of these 4 unknown compounds are in progress. It might be worthwhile to investigate the rate of *methyl*-^{14}C incorporation into these compounds in the various tumors. However, because of the financial problem of injecting large amounts of [*methyl*-^{14}C]methionine into rats, this has not been pursued further. Although the radioactivity incorporation was high, these compounds were not ninhydrin sensitive on the amino acid analyzer. This is probably due to the fact that these compounds exist in extremely minute quantities.

DISCUSSION

One process of posttranslational modification of proteins, protein methylation, is a complex biochemical phenomenon. Various side chains are modified by specific methyltransferases, using *S*-adenosyl-L-methionine as the methyl donor (3, 9, 15, 19). Among these methyltransferases, protein methylase III activity was found always to be elevated whenever cell proliferation was accelerated, *e.g.*, in fetal tissues (10, 17), regenerating adult rat liver (6), and continuously dividing HeLa S-3 cell culture (1, 7). On the other hand, protein methylase III activity in the liver of tadpoles undergoing thyroxine-induced metamorphosis remained unchanged (W. K. Paik, unpublished observation); however, all the biochemical differentiation proceeds in a fixed cell population in this system (2). Therefore, the levels of protein methylase III activity parallel the degree of cell proliferation.

Studies with continuously dividing synchronized HeLa S-3 cell culture, however, indicate that the enzymatic methylation of the ε-amino group of lysine residues of histone is a rather late cellular event; the peak of protein methylase III activity followed the peak of RNA and DNA synthesis (1, 7). Therefore, we postulated earlier that enzymatic methylation of histone molecules is one of the mechanisms that changes the conformational structure of the histone such that the DNA-histone-acidic protein complex can attain a tissue-specific chromatin structure (9, 19).

In this study, we found that the protein methylase III

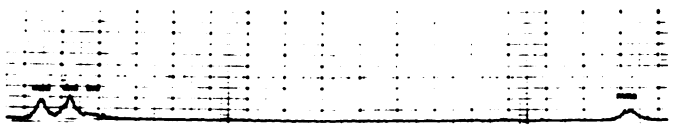

Chart 4. Radiogram of acid hydrolysate of histone isolated from rat liver nuclei. *mml*, ε-*N*-monomethyllysine; *dml*, ε-*N*-dimethyllysine; *tml*, ε-*N*-trimethyllysine; *mma*, N^G-monomethylarginine. Detailed experimental procedures are described under "Materials and Methods."

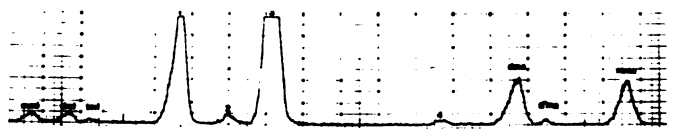

Chart 5. Radiogram of acid hydrolysate of acid-insoluble protein isolated from rat liver nuclei. *mml*, ε-*N*-monomethyllysine; *dml*, ε-*N*-dimethyllysine; *tml*, ε-*N*-trimethyllysine; *dma*, N^G,N^G-dimethylarginine; *d'ma*, N^G,N'^G-dimethylarginine; *mma*, N^G-monomethylarginine. Detailed conditions for *in vitro* methylation and isolation of the acid-insoluble protein, as well as the acid-hydrolysis, are described under "Materials and Methods." *Numbers*, radioactivity peaks the identities of which are not known.

activity in the tumors of fast- and moderately growing rate was elevated (Chart 1), which confirmed our earlier observation (20). Furthermore, ε-alkyllysinase, which has an opposing action to protein methylase III activity *in vitro* (4, 16, 18), decreased in those tumors in which protein methylase III activity was elevated (Chart 2). Thus, the results given in Chart 2 present additional evidence to suggest that protein methylase III and ε-alkyllysinase are physiological antagonists. Strengthening this contention further is the observation that ε-alkyllysinase activity in the host liver of rats bearing the Novikoff hepatoma was depressed, whereas protein methylase III activity was elevated (Chart 3 of Ref. 20; Table 2 of this paper). This decrease seems to be highly specific, since none of the host livers in Table 2 except Morris hepatoma 8995 demonstrate the effect. However, we have not examined the protein methylase III activity in this tumor.

Enzymatic methylation of the guanidino group of arginine residues of protein appears to be potentially important. As shown in Chart 3, the extent of correlation between protein methylase I activity and the rate of tumor growth is much more apparent than in the case of protein methylase III. As in the case of protein methylase III, protein methylase I activity was found to be elevated in all the cell-proliferating systems (1, 6, 7, 17). On the other hand, some dissimilarities were also observed between protein methylases I and III. In a synchronized HeLa S-3 cell culture, the peak of arginine methylation preceded the peak of DNA synthesis while lysine methylation followed DNA synthesis (1). Furthermore, as indicated in Charts 4 and 5, most of the *methyl*-^{14}C incorporated into the H_2SO_4-insoluble protein of isolated rat liver nuclei was found to be associated with methylated arginine derivatives. This is in contrast to the distribution of *methyl*-^{14}C in the acid hydrolysate of histone polypeptides in which the predominant forms of methylated amino acids are in the form of ε-*N*-mono- and ε-*N*-dimethyllysine.

The distribution pattern of various *methyl*-^{14}C-amino acids in the H_2SO_4-insoluble protein of the isolated rat liver nuclei appears to be greatly different from that of calf thymus nuclei (12). In the case of isolated calf thymus nuclei that had been methylated *in vitro* with *S*-adenosyl-L-[*methyl*-^{14}C]methionine, the unknown radioactivity peaks observed in Chart 5 were not detected. Whether these compounds are specific only to the rat nuclei should be investigated.

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