PROTEIN METHYLATION DURING THE DEVELOPMENT OF RAT BRAIN

Woon Ki Paik, Sangduk Kim and Hyang Woo Lee

The Fels Research Institute and Department of Biochemistry,
Temple University School of Medicine, Philadelphia, Pa. 19140

Received December 8, 1971

SUMMARY

The activities of protein methylase I and III are high in the fetal rat brain and decrease rapidly after birth. On the other hand, protein methylase II activity increases slowly during the first 10 days of life and rapidly thereafter. Within 40 days after birth, the levels of all three protein methylase activities reach values corresponding to those of the adult brain. Results obtained for *in vivo* protein methylation indicate that the H_2SO_4-soluble protein is methylated at high rate in fetal brain; this rate then decreases rapidly after birth. Therefore, protein methylation might be involved in the development of the central nervous system.

Methyl substitution of the side chain of protein molecules after genetic translation possibly has a profound effect on the function of proteins (1). Indeed, cells are equipped with various highly specific protein:methyltransferases which methylate protein with S-adenosyl-L-methionine as the methyl donor (2-5). It was of great interest to us to find that brain is one of the richest sources for protein:methyltransferases in the rat (6,7). Furthermore, a variety of methyl substituted amino acids have been found to exist free in human brain (8). We therefore felt it of importance to study the possible relationship between the development of the brain and protein methylati in the rat.

MATERIALS AND METHODS

Materials. S-Adenosyl-L-methionine-methyl-^{14}C (specific activity, 29.9 mC_i/nmole in H_2SO_4, pH 2.0) was purchased from International Chemical and Nuclear Corp., and L-methionine-methyl-^{3}H (specific activity, 147 mC_i/nmole) and DL-methionine-2-^{14}C (specific activity, 6.45 mC_i/nmole) were from New England Nuclear Corp. Histone type II-A from Sigma Chemical Co. (mixture of various histones from calf thymus) was used *in vitro* as the

methyl acceptor for various protein:methyltransferases throughout the experiments. Rats of Wistar strain were used, and were fed ad libitum on Ralston Purina rat chow and housed in a room with 12 hours of light daily.

Enzymatic assay for S-adenosylmethionine:protein methyltransferases. The animals were killed by decapitation at about the same time in the morning to avoid possible diurnal effects on the enzymes. The brains were pooled and were homogenized in 4 volumes of 0.32 M sucrose with a mechanically driven teflon homogenizer, the homogenate was passed through a double layer of cheese cloth, and a portion of the homogenate was centrifuged at 105,000 g for 60 minutes in order to prepare the cytosol fraction. The protein concentration in both whole homogenate and the cytosol was determined by the method of Lowry et al. (9), using bovine serum albumin as standard. They were then diluted with water to give an exact concentration of 10.0 mg protein per ml, because the specific activity of the various protein:methyltransferases differed somewhat depending on the amount of protein used for the enzyme assay. Unless otherwise specified, 1.0 mg of protein (or 0.1 ml of the preparation) was used for the assay. Detailed procedure for determining the activities of protein methylase I (S-adenosylmethionine:protein-arginine methyltransferase), protein methylase II (S-adenosylmethionine:protein-carboxyl methyltransferase) and protein methylase III (S-adenosyl-methionine: protein-lysine methyltransferase) were described elsewhere (7,10).

In vivo protein methylation with doubly labeled methionine. In order to study the methylation of protein in vivo, rat was injected subcutaneously with a doubly labeled methionine solution at the ratio of 0.1 ml solution per 10 g of body weight. One ml of this solution contained 670 μC_i of L-methionine-methyl-^3H and 83 μC_i of DL-methionine-2-^{14}C in normal saline. The reasoning behind this double-labelling experiment will be described later. Two hours later, the animals were decapitated and the brains were homogenized in 0.32 M sucrose solution as 10% homogenate; the homogenate was passed through a double layer of cheese cloth. In the case of young animals, the brains from

Table I

Changes in various protein subfractions during development of brain in rat

Age	Body weight (g)	No of animals pooled	Amount of proetin (mg/100 μg DNA)	Per cent of protein recovered			
				H_2SO_4-insol.	H_2SO_4-sol.	Ethanol-sol.	H_2O-insol.
1 day	6	12	3.09	64.1	14.3	4.7	16.9
5 "	14	6	5.02	71.7	7.5	3.9	16.9
9 "	24	5	8.41	73.6	6.7	4.1	15.6
16 "	48	4	13.25	73.7	6.5	2.9	16.9
26 "	75	7	14.10	76.8	6.3	2.9	14.0
1.6 month	140	4	13.72	76.1	5.8	2.7	15.3

Brains (without cerebellum) were homogenized in 4 times volume of 0.32 M sucrose solution by a teflon homogenizer and the homogenates were passed through a double layer of cheese cloth. Two and half ml of the whole homogenate was processed for determination of DNA and 5.0 ml for protein subfractionation. All procedures are described under Methods.

several rats were pooled. Subfractionation of protein into H_2SO_4-insoluble, H_2SO_4-soluble, ethanol-soluble and H_2O-insoluble fractions was carried out according to the method of Luck et al. (11) (Table I and II). Each fraction

Table II

In vivo methylation of rat brain protein during development

Age (days)	Body weight (g)	Ratio of tritium to carbon-14*				
		whole homog.	H_2SO_4-insol.	H_2SO_4-sol.	ethanol-sol.	H_2O-insol.
1	7	10.2	7.30	12.8	14.0	8.82
7	20	6.55	5.54	8.14	9.20	6.18
26	60	5.43	6.17	6.58	6.92	4.94
50	150	7.13	7.95	9.56	13.2	7.42

Experimental conditions are described under Methods. The protein fractions were treated to remove TCA-soluble radioactivity, nucleic acids and phospholipids (12). Since the step to remove phospholipids involved ethanol-extraction, phospholipids in the ethanol-soluble protein fraction were not removed.
* The ratio in the injected methionine solution was 5.77.

was then treated to remove trichloracetic acid-soluble radioactivity, nucleic acids and phospholipids by the method described previously (12). The remaining doubly labeled protein residue was counted for its radioactivity in 10 ml of Bray's solution (13) with Packard liquid scintillation spectrometer model 3375.

Finally, DNA was determined by the method published (13,14). The enzyme activity is expressed as specific activity which represents μμmoles of S-adenosyl-L-methionine-methyl-^{14}C used/min/mg enzyme protein, and protein concentration was measured by the method of Lowry et al. (9).

RESULTS

The level of various protein methylases in brain during development. Protein methylase I and II are found largely in the cytosol while protein methylase III is exclusively localized in the nuclei (1). Furthermore, it has been shown that there exists in the particulate fractions a specific inhibitor for protein methylase II (15). The enzyme activities were therefore determined in both whole homogenate and in the cytosol.

Fig. 1 illustrates the change in protein methylase I activity during brain development. This enzyme methylates the guanidino group of arginine residues of substrate protein, resulting in the formation of N^G-monomethyl- as well as N^G,N^G-dimethylarginine on acid-hydrolysis of the methylated protein.

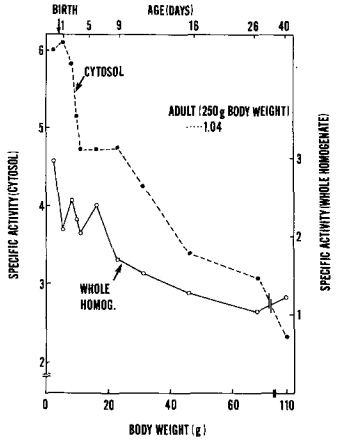

Fig. 1. Protein methylase I activity in developing rat brain All experimental conditions are described under Methods. The brains were pooled from 2-12 rats, depending on the size of the animal.

It can be seen from figure 1 that the specific activity in the cytosol is about 2-fold higher than that found in whole homogenate. It can also be seen that the enzyme activity in fetal brain is quite high, but decreases sharply during the early phase of life span. Thus, the enzyme level in the brain of a rat with a body weight of 110 g (corresponding to about 40 days old) is only about one-third of that found in the fetal brain.

Contrary to the above, protein methylase II activity (this enzyme methylates free carboxyl groups in protein molecules) increases gradually during the first 10 days of life (up to about 25 g of body weight), and thereafter exhibits a rapid increase, reaching to the adult level within 40 days of life span (Fig. 2). It is also noted in figure 2 that the ratio of the enzyme activity in the cytosol to that in whole homogenate is about 2 during the early phase of life, and this ratio increases to about 3 during the adult period. This change in the ratio indicates the presence of natural inhibitor for protein methylase II, and also indicates that the concentration

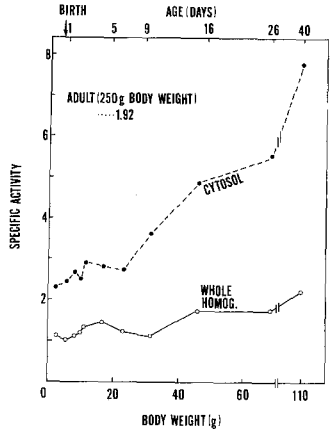

Fig. 2. Protein methylase II activity in developing rat brain The experimental conditions are the same as those of Fig. 1.

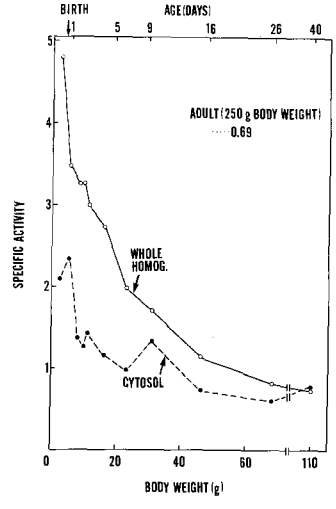

Fig. 3. Protein methylase III activity in developing rat brain The experimental conditions are the same as those of Fig. 1.

of this inhibitor increases as the animal grows.

Fig. 3 illustrates the change in the level of protein methylase III activity in the brain during development. This enzyme is exclusively localized in the nuclei and methylates the ε-amino group of lysine residues in protein molecules. The enzyme activity found in the cytosol is most likely due to breakage of nuclei during homogenization; the specific activity of the enzyme in the cytosol is low, and the amount of enzyme activity recovered in the cytosol is only about 20% of the total enzyme activity (to be published).

The specific activity of protein methylase III is high in the fetal brain and it decreases sharply after birth. During the first 40 days of life span, the enzyme activity corresponds to only one-fifth of those levels found in the fetal brain.

Changes in various protein subfractions during brain development. Table I lists the changes in various protein fractions during brain development. As the animal grows, the amount of protein per cell (or per 100 μg DNA) increases until 2 weeks of life span. Thereafter the amount remains constant (4th column in the table). When the total amount of protein was subfractionated into four subfractions according to their solubilities, H_2O-insoluble and ethanol-soluble proteins were relatively constant in their proportions to the total amount. However, the H_2SO_4-soluble protein decreased significantly during the first 2 weeks of life, while the H_2SO_4-insoluble protei increased correspondingly. It should be noted here that a considerable portion of the H_2SO_4-soluble protein was found to be histone. It is evident from the ta that approximately 75% of the total protein in rat brain is H_2SO_4-insoluble.

In vivo protein methylation In order to study in vivo methylation, rats of vary ing ages were injected subcutaneously with a solution containing L-methionine-methyl-^3H and DL-methionine-2-^{14}C; both of these labeled methionines are incorpc rated into the peptide backbone, and in addition, the methyl-^3H group can also b found to be incorporated onto the side chains. Therefore, the ratio of ^3H to ^{14} indicates the extent of methylation. As shown in Table II, the ratio of ^3H to ^{14}C in the H_2SO_4-insoluble protein is relatively low and does not change signifi cantly during the development of rat brain. On the other hand, the H_2SO_4-solubl the ethanol-soluble and the H_2O-insoluble protein fractions have marked changes the ratio. However, the change in the ratio of the ethanol-soluble fraction mig partly be due to the contamination of this fraction with methylation of phospho-lipids, since this component was not removed due to the technical difficulty. Furthermore, it should also be mentioned that the above ratio does not include t methylation of the free carboxyl groups (by the action of protein methylase II), because of the instability of the carboxymethyl ester in hot TCA-extraction meth

used to remove nucleic acids.

DISCUSSION

We have demonstrated in the present paper that the enzymes which methylate the guanidino group of arginine and the ε-amino group of lysine residues in histone molecule are high in the fetal brain, and that these enzyme activities start to decrease immediately after birth. On the other hand, we have also shown that the enzyme which methylates the free carboxyl groups in protein molecules starts to increase shortly after birth. All three enzymes reached those levels of adult brain in about the first 40 days of life span. These facts indicate roles for these enzymes in development of the central nervous system. However, it is of interest to note that these activities are not at their highest during the time when myelinization of the developing rat brain occurs (about 10 days after birth).

The results of *in vivo* methylation of protein with doubly labeled methionine indicate that the rate of protein methylation was high in the fetal brain and decreased immediately after birth, thus confirming the results obtained by enzymatic assay. Although the H_2SO_4-insoluble protein comprises approximately 75% of the total protein in rat brain, the rate of *in vivo* methylation is not too high. Contrary to the H_2SO_4-insoluble protein, the H_2SO_4-soluble protein is methylated at a high rate in fetal brain, however this rate decreases sharply during early age of rat. Even though the rate of methylation is low in the H_2SO_4-insoluble protein, the total amount of methylated amino acids should be very high because of the large mass in the brain. More detailed investigations concerning the nature of each methylated amino acid as well as the further characterization of the H_2SO_4-insoluble and H_2SO_4-soluble proteins are in progress.

REFERENCES

1. Paik, W. K., and Kim, S., Science, 174, 114 (1971).
2. Paik, W. K., and Kim, S., J. Biol. Chem., 243, 2108 (1968).
3. Kim, S., and Paik, W. K., J. Biol. Chem., 245, 1806 (1970).
4. Liss, M., Maxam, A. M., and Cuprak, L. J., J. Biol. Chem., 244, 1617 (1969).
5. Paik, W. K., and Kim, S., J. Biol. Chem., 245, 6010 (1970).
6. Paik, W. K., and Kim, S., J. Neurochem., 16, 1257 (1969).
7. Paik, W. K., Lee, H. W., and Lawson, D., Exp. Geront., 6, 271 (1971).
8. Kakimoto, Y., and Akazawa, S., J. Biol. Chem., 245, 5751 (1970).
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).
10. Paik, W. K., Lee, H. W., and Morris, H. P., Cancer Res., in the press (1972).
11. Luck, J. M., Rasmussen, P. S., Satake, K., and Tsvetikov, A. N., J. Biol.

Chem., 233, 1407 (1958).
12. Allfrey, V. G., Mirsky, A. E., and Osawa, S., J. Gen. Physiol., 40, 451 (1957).
13. Schmidt, G., and Thannhauser, S. J., J. Biol. Chem., 161, 83 (1945).
14. Dische, Z., in The Nucleic Acids, (ed. by E. Chargaff and J. N. Davidson), Academic Press, New York, Vol. I, (1955), p.287.
15. Kim, S., and Paik, W. K., Biochim. Biophys. Acta, in the press (1971).

ACKNOWLEDGEMENT

This work was supported by research grants AM 09603 from the National Institute of Arthritis and Metabolic Diseases, BG 22663 from the National Science Foundation, CA 12226 and 10439 from the National Cancer Institute and 1-P01-HD-05874-01 from National Institute of Child, Health and Human Growth.