ENZYMATIC DEMETHYLATION OF CALF THYMUS HISTONES

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Summary

Enzymatic demethylation of ε-N-methyllysine residues of calf thymus histone has been investigated. Kidney contains the highest enzyme activity among the rat organs tested, and the enzyme activity is associated with the nuclear and mitocondrial fractions. Formaldehyde-C-14 is the product of demethylation of enzymatically prepared histone-methyl-C-14. Evidence indicates that demethylation occurs on the intact histone molecule.

The enzymatic methylation of a protein molecule subsequent to the formation of peptide bonds is a rather common biochemical phenomenon (1). The ε-amino group of lysine, guanidino group of arginine, and carboxyl group of dicarboxylic amino acid residues of protein molecules are methylated by separate enzymes utilizing S-adenosyl-L-methionine as the methyl donor (2-6). The enzymatic methylation of the ε-amino group of lysine residues, however, seems to be of the most interest; protein methylase III (S-adenosylmethionine:protein-lysine methyltransferase) which is responsible for the methylation is localized in the nucleus, and the enzyme activity is elevated whenever cell proliferation is accelerated such as in fast growing hepatomas (7), fetal brain (8), regenerating rat liver (9-11), testis of young rat (12) and continuously dividing HeLa S-3 cell culture (13). Furthermore, protein methylase III appears to be highly specific for histone in the synchronized HeLa S-3 cell culture (14). A question arises therefore concerning the turnover of the methyl group incorporated into the protein. We have recently presented evidence to indicate that approximately 2% of the incorporated methyl groups are turning over per hour in HeLa S-3 cell culture (13). However, there are also reports to contradict the above contention (15,16). In the present communication, evidence is presented to demonstrate the presence of a demethylating enzyme in rat kidney.

Materials and Methods

Materials S-Adenosyl-L-methionine-methyl-^{14}C (specific activity, 28.9 mC_i/mmole

in an aqueous solution, pH 3.0) was purchased from Amersham/Searle Corp.; histone type II-A (a mixture of various histone fractions from calf thymus) from Sigma Chemical Co.; 5,5-dimethyl-1,3-cyclohexanedione (dimedon) from Eastman Kodak Co.; and semicarbazide hydrochloride from Fisher Chemical Co. All remaining chemicals were obtained from either Sigma Chemical Co. or from various local sources.

Preparation of enzymatically methylated histone-methyl-^{14}C Approximately 40 g of frozen calf thymus were thawed, and homogenized in 9 times their volume of 0.25 M sucrose plus 0.003 M $CaCl_2$ solution by an electrically driven teflon glass homogenizer. The homogenate was filtered through a double layer of cheesecloth and centrifuged at 700 g for 10 minutes. The pellet was rehomogenized in 20 ml of the above sucrose solution. The crude nuclear fraction thus obtained (a little more than 20 ml) was incubated with 10 ml of S-adenosyl-L-methionine-methyl-^{14}C (478 mμmoles; 2.58 x 10^7 cpm), 10 ml of 0.5 M Tris-HCl buffer at pH 9.0 and 10 ml of histone type II-A (30 mg) at 37^0 for 3 hours. At the end of the incubation, 6 ml of 2 N H_2SO_4 was added to the incubation mixture which was then centrifuged at 39,000 g for 10 minutes. The precipitate was further extracted three times with 0.2 N H_2SO_4. All the extracts were combined, and more than two and half times volume of 95% ethanol was added to the extract. The mixture was left in the cold room overnight, and the precipitate was collected by centrifugation at 39,000 g for 10 minutes. The pellet was extracted with water a few times, and a sufficient amount of 0.1 M $BaCl_2$ was added into the water-extract in order to remove the sulfate ion. The $BaCl_2$-treated sample was then dialyzed against 6,000 ml of H_2O, and the water was changed once. The final product had a specific activity of 1.53 x 10^4 cpm per mg protein, and,as shown in Table III, all the radioactivity was found as ε-N-mono, ε-N-di, and ε-N-trimethyllysine.

Enzymatic assay for demethylation of histone-methyl-^{14}C Assay for the enzymatic demethylation of histone-methyl-^{14}C primarily consisted of determining the liberated $H^{14}CHO$ as formaldemethone after reacting the 25% trichloracetic acid (CCl_3COOH) soluble fraction of the incubation mixture with dimedon. One tenth to one half of one ml of whole homogenate was incubated with 0.3 ml of histone-methyl-^{14}C

(17,500 cpm), 0.2 ml of 0.1 M semicarbazide (trapping agent for $H^{14}CHO$; pH of the water solution was adjusted to about neutrality with 0.5 N NaOH), 0.2 ml of 0.5 M phosphate buffer at pH 7.2 and water to bring the final volume to 1.2 ml. The incubation was carried out at 37° for the indicated period of time, and the reaction was terminated by addition of 1.2 ml of 50% CCl_3COOH. The control was prepared either by adding the enzyme preparation after the addition of CCl_3COOH or by incubating enzyme preparation heated at 100° for 5 minutes. The supernatant at 105,000 g for 20 minutes was then reacted with 60 ml of 0.4% dimedon according to the method of Frisell and Mackenzie (17). One ml of non-radioactive formaldehyde (0.74%; 8.0 mg) was added as carrier, giving 78 mg of theoretical yield of formaldemethone. The above dimedon-treated samples were left at room temperature overnight, and the precipitates were collected by filtration. The amount of formaldemethone ranged around 69.2 ± 0.7 mg (n=10). The formaldemethone was recrystallized from hot ethanol, and the radioactivity from approximately 10 mg was measured in 10 ml of Bray's solution (18). A large amount of formaldemethone quenched the radioactivity extensively. From the radioactivity obtained in the 10 mg portion, the total radioactivity was calculated. The enzyme activity was expressed as μμmoles of $H^{14}CHO$ formed/hour/mg protein, and the protein concentration was determined by the method of Lowry et al. (19). Finally, fractionation of subcellular fraction of rat kidney was carried out according to the method described (20).

Results and Discussion

Identification of the demethylation product as formaldehyde When the incorporated $^{14}CH_3$ groups to the ε-amino group of lysine residues were liberated, either $^{14}CH_3OH$ or $H^{14}CHO$ will result. However, a distillation method to detect $^{14}CH_3OH$ (21) did not show any trace of radioactive methanol in the incubation mixture in which the rat kidney whole homogenate and histone-methyl-^{14}C were incubated for 3 hours at 37°. However, the radioactivity found in 25% TCA-soluble fraction was recovered as formaldemethone. The specific radioactivity in formaldemethone (dpm/10 mg of formaldemethone) remained constant during repeated recrystallizations. For example, the first recrystallization from hot ethanol gave 316 dpm and the second 326 dpm/

10 mg of formaldemethone. Furthermore, the melting point of formaldemethone resulting from the incubation mixture (145-155°C) coincided with that of the authentic compound (148-154°C), whereas the melting point of dimedon was 125-128°C.

Effect of incubation time Fig. 1 illustrates the effect of incubation time on the demethylation activity in rat kidney and liver. The demethylation activity is proportional to the period of incubation time.

Effect of enzyme concentration Fig. 2 shows the effect of enzyme (kidney whole

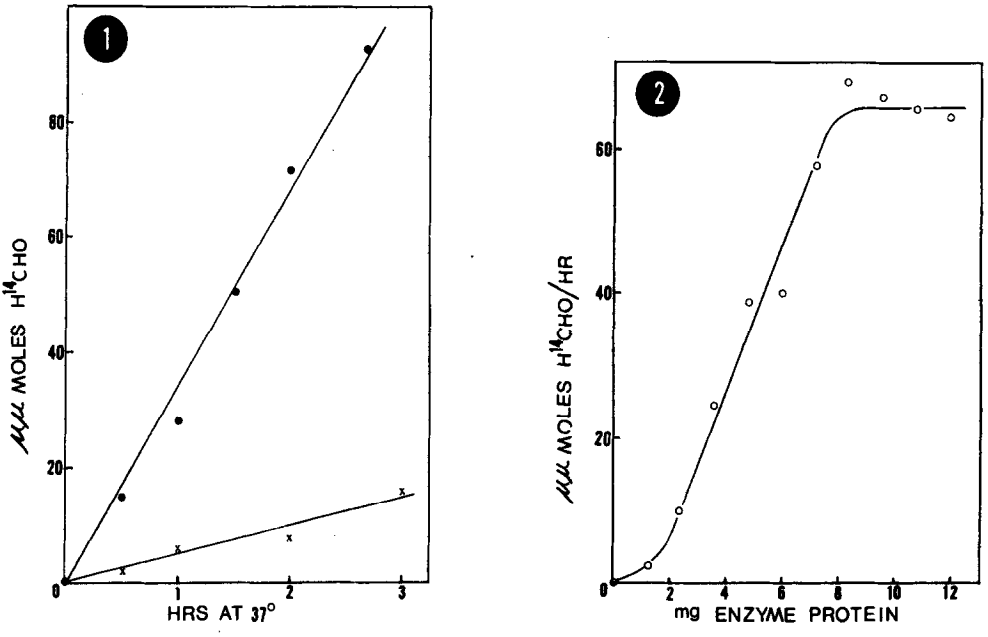

Fig. 1. Effect of period of incubation time All the experimental conditions are described under Methods. ●—● represent rat kidney (5.73 mg of protein) and x- -x for liver (18.5 mg of protein). One μμmole of formaldehyde corresponds to 54 cpm.

Fig. 2. Effect of enzyme concentration Rat kidney whole homogenate was used. The rest of the experimental conditions are described under Methods.

homogenate) concentration on the demethylation activity. The curve has a sigmoidal shape, indicating either the presence of an allosteric affector or a simple activator. The plateau with the high enzyme concentration might simply be due to the limiting amount of the substrate histone-methyl-^{14}C. This seems to be worthy of

reinvestigation when the purified enzyme preparation becomes available.

Distribution of the demethylation activity in various rat organs Table I lists the results on the distribution of the demethylation activity in various rat organs. As seen in the table the kidney has the highest enzyme activity among the organs examined, and the liver has only about 10% of the activity found in the kidney.

Table I

Demethylation Activity in Various Organs of Rat

Organs	Amount of protein used in incubation (mg)	Specific activity (μμmole of HCHO-C-14/hr/mg protein
Kidney	5.7	6.49
Liver	21.7	0.65
Testis	10.0	0.64
Lung	9.8	0.18
Spleen	18.2	0.03
Thymus	10.5	0.03
Brain	19.9	0.01
Pancreas	15.3	0

Various tissues were rinsed once with a large amount of cold water, and were homogenized in 4 times volume of water by an electrically driven teflon glass homogenizer. The whole homogenate was passed through a double layer of cheesecloth. Half a ml of the whole homogenate, except kidney where 0.2 ml was used, was incubated with the reagents described under Methods. The rest of the experimental procedures are described under Methods.

Subcellular distribution of the demethylation activity in rat kidney As shown in Table II, practically all of the demethylation activity in rat kidney is localized in the nuclear and mitochondrial fractions. Although the specific activity of the demethylation activity is higher in the mitochondrial fraction, almost an equal amount of the total activity is found in these two subcellular fractions.

Identity of the demethylation activity We have previously reported the existence

Table II

Subcellular Distribution of the Demethylation Activity in Rat Kidney

Fraction	Volume	Protein			Enzyme activity		
		Amount	Total	Recovery	Specific act.*	Total*	Recovery
	(ml)	(mg/ml)	(mg)	(%)			(%)
Whole homogenate	4.0	37.2	148.8	100.0	7.50	1,116	100.0
Nuclear	3.1	12.8	39.7	26.7	10.63	422	37.8
Mitochondrial	2.6	11.8	30.7	20.6	15.48	475	42.6
Microsomal	2.6	8.6	22.4	15.1	3.71	83	7.5
Cytosol**	3.6	10.5	37.8	25.4	0	0	0

* Specific activity represents μμmole of formaldehyde formed/hour/mg protein, and total enzyme activity μμmole formaldehyde formed/hour.

** The cytosol fraction was obtained by centrifuging the whole homogenate at 105,000 g for 60 minutes. This procedure eliminated dilution of the final cytosol fraction due to the washings of other subcellular fractions. Two-tenth ml of whole homogenate and 0.5 ml each of the other fractions were used for the enzyme assays. Incubation period was 1 hour. The rest of the experimental procedures are described under Methods.

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of an enzyme ε-alkyllysinase (ε-Alkyl-L-lysine:Oxygen Oxidoreductase; E. C.:1,5,3,a) which dealkylated free ε-N-monomethyl-L-lysine and ε-N-dimethyl-L-lysine with an oxygen consumption of 0.5 mole to give an equivalent of L-lysine and formaldehyde (22). The enzyme was found in its highest amount in the rat kidney and was associated with mitochondria. Furthermore, ε-alkyllysinase could demethylate α-keto-ε-methylaminocaproic acid, indicating that the α-amino group of ε-N-methyl-lysine is not essential for the reaction. This further suggested a possibility of demethylation of protein molecule by ε-alkyllysinase. Therefore, it is of great importance in the present study to examine if the histone-methyl-^{14}C was first hydrolyzed to give rise in the formation of free ε-N-methyl-L-lysine(methyl-^{14}C) and the latter subsequently demethylated by ε-alkyllysinase.

Therefore, histone-methyl-^{14}C was first reacted with rat kidney homogenate, the histone was re-isolated, and the distribution of the radioactive methyl groups in the hydrolyzate of the protein was examined (Table III). The control histone

Table III

Analysis of Radioactive Amino Acids in Histone-methyl-^{14}C treated with Rat Kidney Homogenate

Histone-methyl-^{14}C	Amount of L-lysine (μmole)	Radioactivity MML* (cpm)	DML (cpm)	TML (cpm)	Total (cpm)
Control	10.0	16,190	13,343	3,682	33,215
Treated	10.0	4,601	3,520	0	8,121

* MML, DML and TML represent ε-N-monomethyllysine, ε-N-dimethyllysine and ε-N-trimethyllysine, respectively.

Six tenth ml of kidney whole homogenate (17.8 mg of protein), 1.0 ml of histone-methyl-C-14 (0.58 x 10^5 cpm), 0.4 ml of 0.5 M phosphate buffer of pH 7.2 and 0.4 ml of water were incubated at 37°C for 4 hours. The reaction was stopped by addition of sulfuric acid to a final concentration of 0.25 M. The control was prepared by incubating all of the components except histone-methyl-C-14, and then by adding the histone after sulfuric acid. Both the control and the treated samples were then treated in order to extract histone according to the method described under Methods. After dialysis, the isolated histones were hydrolyzed in 6 N HCl for 85 hours at 110°C in vacum-sealed tubes, and the acid-hydrolyzates were analyzed by Perkin-Elmer KLA-3B amino acid analyzer with Packard Tri-Carb liquid flow cell scintillation spectrometer.

contained most of the methyl-^{14}C among various ε-N-methylated lysine derivatives. However, when the histone-methyl-^{14}C was reacted with rat kidney homogenate, approximately 80% of the methyl-^{14}C was removed (the values in the table are expressed on the basis of unit amount of L-lysine). The result in Table III therefore strongly indicates that the presently described demethylation activity occurred mainly at the protein level.

When the kidney whole homogenate was treated at 100°C for 5 minutes, the demethylation activity was completely abolished. Therefore, the demethylation activity presented in the present communication was most likely due to an enzyme. However, the identity of the present demethylase with ε-alkyllysinase was not quite certain; both enzymes were present in high concentration in the rat kidney, and were particulate-bound, even though the former in the nuclear and mitochondria and the latter in the mitochondrial (22). There was a possibility that ε-alkyllysinase might demethylate histone-methyl-^{14}C as well as free ε-N-methyllysines. We are now in the process of further purification of demethylase as well as

ε-alkyllysinase. It should be noted here that ε-alkyllysinase was purified only 3-fold (22).

Consideration of turnover of the incorporated methyl groups is of great importance in the study on the biochemical significance of protein methylation. If the methyl group does not turnover, this indicates that protein methylation might be a process which modifies protein without additional metabolic activity. However, we have previously shown that approximately 2% of the total methyl groups are removed per hour during the synchronized HeLa S-3 cell culture (13). Together with the presently described demethylase, these results indicate that protein methylation is a dynamic process, and not a terminal "sink" reaction.

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