In Vitro Studies on the Methylation of Histones in Rat Brain Nuclei*

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When isolated nuclei from 12-day-old rat brains were incubated with S-adenosyl-L-[methyl-3H]methionine, significant amounts of H-methyl were incorporated into lysyl residues in histones H3 and H4. About 0.02% of the total methylation sites on histone H3 and 0.019% of the sites on histone H4 were unmethylated at the time the nuclei were isolated. Methylation of these sites proceeded stepwise, progressing to a stable ratio of 0.93:1.0:0.17 for N'-mono-, N'-di-, and N'-trimethyllysine in histone H3 and 0.19:1.0 for N'-mono- and N'-dimethyllysine in histone H4. The Kₐ values of the enzyme for S-adenosyl-l-methionine were 11.5 ± 1.1 µM and 12.5 ± 1.3 µM with histones H3 and H4 as methyl acceptors, respectively. The Vₘₐₓ values were 11.1 and 5.3 pmol of H-methyl incorporated/min/mg of histone H3 and H4, respectively. Since histone H3 contains 2 mol of N'-methyllysinelmol and histone H4 contains 1 mol/mol, no difference in the overall rates of methylation can be deduced from the data. S-Adenosyl-L-homocysteine, one of the products of the reaction, was a competitive inhibitor with respect to S-adenosyl-l-methionine. The Kₐ values for S-adenosyl-l-homocysteine were 5.5 ± 0.4 µM and 5.9 ± 0.5 µM with histones H3 and H4 as methyl acceptors, respectively.

It is now well established that specific lysyl residues on the arginine-rich histones are methylated. Histone H4 from calf thymus contains a single methylated lysyl residue at position 20, while histone H3 from calf thymus or carp testis contains methylated lysyl residues at positions 9 and 27 (1-3). Both sites on histone H3 contain N'-mono-, N'-di-, and N'-trimethyllysine, while N'-trimethyllysine is absent from histone H4. Honda et al. (4) have obtained similar results for the methylated lysyl residues on histone H3 and H4 as methyl acceptors, respectively. The Vₘₐₓ values were 11.1 and 5.3 pmol of H-methyl incorporated/min/mg of histone H3 and H4, respectively. Since histone H3 contains 2 mol of N'-methyllysine/mol and histone H4 contains 1 mol/mol, no difference in the overall rates of methylation can be deduced from the data. S-Adenosyl-L-homocysteine, one of the products of the reaction, was a competitive inhibitor with respect to S-adenosyl-l-methionine. The Kₐ values for S-adenosyl-l-homocysteine were 5.5 ± 0.4 µM and 5.9 ± 0.5 µM with histones H3 and H4 as methyl acceptors, respectively.

The methylation sites and amino acid sequences of histone H3 and H4 are highly conserved (1-13). However, there appear to be differences in the extent of methylation of lysyl residues at these sites. This is particularly striking in the pea, where histone H4 is entirely unmethylated and trimethyllysine is absent from histone H3 (6, 14-16). Some of the differences in methylation appear to be genetic, while others may be related to the mitotic state of the cells at the time the histones were isolated from a particular organ. In HeLa cells, methylation occurs mainly in the late S cycle (17, 18). The ratio of N'-mono- to N'-dimethyllysine in the malignant cell was about twice that found in the normal cell. In the rat, the extent of methylation of the arginine histones does not vary from organ to organ, but varies significantly with age (19). In histone H3 from 10-day-old rats, the molar ratio of mono-di-trimethyllysine was 0.55:1.0:0.35. In histone H4 from these same age animals, the molar ratio of mono-di-dimethyllysine was 0.1:0.9. These ratios shift towards the more highly methylated forms with age. If methylation of histones is a late event occurring after the histones are transported into the nuclei, then the newly synthesized polypeptide chains should not be fully methylated in rapidly proliferating tissue.

In this communication, we employed nuclei from the brain of young rats in order to determine: (a) the number of unmethylated lysyl residues in histones, (b) if methyl groups are added sequentially or simultaneously, (c) if histones are methylated prior to or after they are bound to DNA, and (d) some of the kinetic properties of the histone-lysine methyltransferase.

**EXPERIMENTAL PROCEDURES**

**MATERIALS** - S-Adenosyl-L-[methyl-3H]methionine (1.8 Ci/mmol) were purchased from International Chemical and Nuclear Corp. S-Adenosyl-L-[methyl-3H]methionine was diluted to 1.0 Ci/ml with unlabeled S-adenosyl-L-methionine prepared by the method of Schlenk and Depalma (20). [3H]lysine (7.0 Ci/ml) was obtained from Schwarz/Mann. S-Adenosyl-L-homocysteine, S-adenosyl-l-homocysteine sulfoxide, and S-riboyl-l-homocysteine were prepared by the method of Duerre et al. (21). S-Adenosyl-p-homocysteine was a gift from Dr. Fritz Schlenk, Argonne National Laboratories, Argonne, Ill. S-Adenosyl-thio-α-ketobutyrate was prepared enzymatically from S-adenosyl-l-homocysteine using the L-amino-acid oxidase from Proteus rettgeri (22) and purified according to the procedure of Duerre et al. (23). Methylthioadenosine was prepared following the procedure of Schlenk and Ehninger (24). L-Homocysteine was prepared from L-homocysteine thiolactone with alkali (25). Cyclic adenosine 3′,5′-monophosphoric acid was obtained from Sigma Chemical Co.

**Preparation of Nuclei** - Long-Evans rats, 12 to 14 days old, were killed by decapitation and their brains were removed. The brains were placed in cold 0.32 M sucrose containing 1.0 mM MgCl₂ and 1.0 mM potassium phosphate buffer, pH 7.8 (buffered sucrose). The brains were homogenized and the crude nuclei were harvested by centrifugation (26). The crude nuclei were washed three times in buffered sucrose by repeated centrifugation and suspended in the same solution at a concentration of about 5 mg of DNA/ml.

**In Vitro Methylation of Histones** - Reaction mixtures containing nuclei (20 mg of DNA). 15.0 µCi S-adenosyl-L-[methyl-3H]methionine (1.0 Ci/mmol), 0.32 m sucrose, 1.0 mM MgCl₂, and 1.0 mM potassium phosphate buffer, pH 7.8, were incubated at 37° for designated time periods. The final pH of reaction mixture was 6.8. The reaction was

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terminated by adding S-adenosyl-L-homocysteine to a final concentration of 10 μM and chilling to 0° on a dry ice/alcohol bath. The nuclei were lysed by adding 5 volumes of cold phosphate buffer, pH 7.6. The chromatin was harvested by centrifugation and washed twice, each with 20 mM phosphate buffer, methanol:chloroform (1:2), methanol, acetone, and acetic acid, pH 3.0 (29). The histones were extracted with 0.4 M HCl, precipitated with 5 volumes of acetone, and harvested by centrifugation. The histones were dissolved in 0.01 M HCl, 1.0 mM dithiothreitol, and 6.0 M urea. The histones were fractionated on Bio-Gel P-10 as described in Fig. 1. Proteins under the peaks were quantitated by the method of Lowry et al. (27) using a Technicon auto analyzer. Radioactivity under peaks was determined by placing 0.2-ml aliquots of each fraction in 10 ml of scintillation fluid and counting in a Packard Tri-Carb scintillation spectrometer (28). Fractions under the peaks with uniform specific activities were pooled and concentrated by lyophilization. Polyacrylamide gel electrophoresis based on the method of Panyim and Chalkley (29) was used to check the purity of the histone fractions. From 1.0 to 2.0 mg of histone H3 and H4 were hydrolyzed and the basic amino acid composition was ascertained on Beckman PA-35 resin as previously described (19).

Similar experiments were attempted using brain nuclei purified by centrifugation through 1.77 M sucrose buffer. However, nuclei so prepared were difficult to resuspend in either 0.32 or 1.77 M sucrose and had a tendency to lyse upon incubation at 37°C.

Preparation of Chromatin - Crude nuclei were prepared from 12-to 14-day-old rats as described above. After purification by centrifugation through 1.77 M sucrose, the nuclei were lysed with 20 mM phosphate, pH 7.6. The chromatin was harvested by centrifugation and washed twice with the same buffer. The chromatin was suspended in 30 mM phosphate buffer (final pH 6.8) at a concentration of approximately 5 mg of DNA/ml. DNA was quantitated by the method of Burton (30).

**RESULTS**

When isolated rat brain nuclei were incubated with S-adenosyl-L-[methyl-3H]methionine, significant amounts of H3-methyl groups were incorporated into histones H3 and H4 (Fig. 1). There was a measurable amount of radioactivity associated with a protein which eluted just after histone H1. This may be the result of dimerization of histone H3; however, this has not been fully established.

The time course of incorporation of H3-methyl groups into histones H3 and H4 in intact nuclei is presented in Fig. 2. Methylation of these histones proceeded at a linear rate for 10 to 15 min, reaching saturation after 40 min. The further addition of S-adenosyl-L-[methyl-3H]methionine at this point has no effect; therefore, all available sites appeared to be fully methylated. Methylation of histone H3 proceeded about 2.6 times faster than methylation of H4.

The distribution of methylated basic amino acid residues was ascertained by amino acid analysis. All the radioisotope incorporated into histones H3 and H4 occurred as [methyl-3H]N-methyllysines. There was no radioactivity detectable in the regions where methylarginine or methylhistidine eluted from the column. The rates of incorporation of H3-methyl groups into N-methyllysine from histones H3 and H4 are presented in Fig. 3. Monomethyl groups were added to both histones at a rapid linear rate, then decreased with time. There was a short lag prior to the formation of N'--dimethyllysine in both histones, while there was a much more pronounced lag prior to the formation of N''-trimethyllysine in histone H3. In histone H3, the ratio of mono-di-trimethyllysine shifted toward the higher methylated lysine forms with time (Table I). After 40 min, the ratio approached that which we had previously observed in vivo (0.55:1.0:0.35); however, there appeared to be somewhat more N''-monomethyllysine in the in vitro experiment. As with histone H3, the ratio of mono- to dimethyllysine in histone H4 shifted toward the higher methylated forms (Table II). The ratio after 40 min approached that which we had previously observed in vivo (19). From the above data, it appeared that methylation proceeded stepwise, that is mono- to di- to trimethyllysine in histone H3 and mono- to diethyllysine in histone H4.

At saturation, 0.48 and 0.13 mmol of H3-methyl were incorporated/mol of histone H3 and H4, respectively. The total amount of N'-methyllysine was 2.0 mol/mol of histone H3 and 1.0 mol/mol of histone H4. From the radioisotope data, it can be calculated that 0.024% of the histone H3 molecules and 0.013% of the histone H4 molecules remained unmethylated at the time the animals were killed. This probably represents newly synthesized histone which has not as yet been methylated.

The unmethylated histones could possibly be free in the nucleoplasm. However, washed chromatin, when incubated...
with S-adenosyl-L-[methyl-3H]methionine at pH 6.8 for 120 min, incorporated 74 and 32 pmol of 3H-methyl/mg of histone for histones H3 and H4, respectively. These results are comparable to the data obtained for nuclei (Fig. 2). From these data, it appears that the enzyme is firmly bound to chromatin and histones are methylated after binding to the chromatin.

Kinetics - In intact nuclei, the $K_m$ of the enzyme for S-adenosyl-l-methionine with histone H3 as methyl acceptor was 12.5 $\pm$ 1.3 $\mu M$ and with histone H4 as methyl acceptor the $K_m$ for S-adenosyl-l-methionine was 11.5 $\pm$ 1.1 $\mu M$ (Fig. 4). The $V_{max}$ was 11.1 pmol of 3H methyl incorporated/min/mg of histone H3. This value was about twice that observed with histone H4 (5.3 pmol of 3H methyl/min/mg). Since histone H3

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**Table I**

| Incubation time (min) | Mono-lysine (methyl-3H) | Dimethyl-lysine (methyl-3H) | Trimethyl-lysine (methyl-3H) | Total (methyl-3H) | Ratio (mono:-:di-trimethyl-lysine) | Histone H3 (mg) |
|----------------------|------------------------|-----------------------------|----------------------------|------------------|----------------------------------|----------------|
| 5                    | 68                     | 35                          | 3                          | 106              | 1.95:1:0:0.08                    | 15,200         |
| 10                   | 141                    | 80                          | 7                          | 228              | 1.76:1:0.09                      | 11,280         |
| 15                   | 167                    | 129                         | 17                         | 313              | 1.29:1:0.13                      | 11,280         |
| 25                   | 249                    | 208                         | 36                         | 488              | 1.23:1:0.18                      | 11,280         |
| 40                   | 210                    | 227                         | 31                         | 468              | 0.93:1:0.14                      | 11,280         |
| 80                   | 220                    | 237                         | 39                         | 496              | 0.93:1:0.17                      | 11,280         |

*Values are given in molar ratios calculated by multiplying the values in Fig. 3 by the molecular weight of histone H3 (15,200). To convert from micromoles of 3H methyl incorporated to micromoles of [methyl-3H]dimethyllysine, the values were divided by 2.*

**Table II**

| Incubation time (min) | Mono-lysine (methyl-3H) | Dimethyl-lysine (methyl-3H) | Trimethyl-lysine (methyl-3H) | Total (methyl-3H) | Ratio (mono:-:di-trimethyl-lysine) | Histone H4 (mg) |
|----------------------|------------------------|-----------------------------|----------------------------|------------------|----------------------------------|----------------|
| 5                    | 18                     | 52                          | 70                         | 0.34:1.0          |                                  | 11,280         |
| 10                   | 24                     | 72                          | 105                        | 0.46:1.0          |                                  | 11,280         |
| 15                   | 21                     | 86                          | 107                        | 0.24:1.0          |                                  | 11,280         |
| 25                   | 20                     | 103                         | 123                        | 0.19:1.0          |                                  | 11,280         |
| 40                   | 19                     | 111                         | 130                        | 0.17:1.0          |                                  | 11,280         |
| 80                   | 18                     | 104                         | 123                        | 0.17:1.0          |                                  | 11,280         |

*Values are given in molar ratios calculated by multiplying the values in Fig. 3 by the molecular weight of histone H4 (11,280). To convert from micromoles of 3H methyl incorporated to micromoles of [methyl-3H]dimethyllysine, the values were divided by 2.*

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**Fig. 4.** Plots of reciprocal of reaction velocity ($1/V$) against reciprocal of micromolar concentration of S-adenosyl-L-[methyl-3H]methionine in the absence (○○○) and presence (×××) of 4.0 $\mu M$ S-adenosyl-l-homocysteine. Reaction mixtures contained whole rat brain nuclei (6 mg of DNA), 0.32 M sucrose, 1.0 mM MgCl₂, 20 mM phosphate buffer (final pH 6.8), and varied concentrations of S-adenosyl-L-[methyl-3H]methionine. After 4 min, the reaction mixtures were made 10.0 $\mu M$ with respect to S-adenosyl-l-homocysteine and chilled to 0°. The histones were prepared and fractionated as outlined under Fig. 1.
In Vitro Methylaion of Histones

contains two methylation sites and histone H4 only one site, no difference in overall rates of methylation of the two histones can be deduced from this data.

The effect of several compounds on the rate of methylation of histones H3 and H4 was measured. Of all the compounds tested, including S-adenosyl-
L-thio-α-ketobutyrate, S-riboyl-
L-homocysteine, S-adenosyl-L-homocysteine sulfoxide, adenosine, adenine, methylthioadenosine, AMP, ATP, homocysteine, homoserine, and methionine, only the S and L isomers of S-adenosylhomocysteine had any effect on that rate of methylation of histones H3 and H4. Inhibition of the enzyme by S-
adenosyl-L-homocysteine was of the competitive type with respect to S-adenosyl-L-methionine (Fig. 4). The inhibition constants (Kᵢ) for S-adenosyl-L-homocysteine were 5.5 ± 0.4 μM and 5.9 ± 0.5 μM with H3 and H4 as methyl acceptors, respectively.

**DISCUSSION**

One of the major difficulties in studies on the in vitro methylation of histones is obtaining a suitable substrate. Histones H3 and H4 do not turn over in adult brain, nor do the methyl groups turn over independently of the polypeptide chain (31). The irreversibility of methylation of histones has also been reported in other tissues (32, 33). Consequently, histones from such tissues should be fully methylated. This has been substantiated in the brain by both in vitro and in vivo studies. When brain nuclei from adult animals were incubated with radiolabeled S-adenosyl-L-methionine, histones H3 and H4 failed to incorporate significant amounts of labeled methyl groups (34). Furthermore, when adult rats are given radiolabeled lysine and methyl labeled methionine, only trace quantities of radioactivity were incorporated into brain histones. In contrast, significant amounts of H-Methyl groups were incorporated into histones when the nuclei were prepared from brains of young rats (Fig. 2). Even in these nuclei, the number of unmodified lysyl residues in histones H3 and H4 are quite limited (Tables I and II). Apparently a small fraction of the cells are in a state in which the newly synthesized histones have condensed with DNA, but are not yet fully methylated.

Following partial hepatectomy, methylation of histones was found to be a late event occurring for a significant time after DNA synthesis (35, 36). In tissue culture, methylation occurs following partial hepatectomy, methylation of histones was found to be a late event occurring for a significant time after DNA synthesis (35, 36). In tissue culture, methylation occurs.

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difference in the \( K_m \) values of the enzyme for S-adenosyl-L-methionine with histone H3 and H4 as methyl acceptor, nor was there any significant difference in the \( K_m \) values for S-adenosyl-L-homocysteine with the two histones as methyl acceptors.

The differences in the extent to which the lysyl residues in both histones H3 and H4 are methylated may be the result of more than one histone methyltransferase. However, it is more probable that the extent to which these lysyl residues are methylated is conferred by the arrangement of the histones on the chromatin. Similarities in methylation sequences, -X-Arg-X- common to both histones H3 and H4 and -Ala-Arg-Lys-Ser- common to both sites in histone H3, have been observed by DeLange et al. (1, 2). Loss of specificity of the enzyme for specific recognition sites on soluble histones is evident by the fact that free histones from the brains of old rats are as good methyl acceptors as the histones from young rat brains when incubated with soluble enzyme (34). Loss of specificity is also evident by the finding that free lysine-rich and slightly lysine-rich histones will accept methyl groups in a soluble system (34, 42-44). These histones do not accept methyl groups when bound to chromatin in the presence of soluble enzyme (44). The loss of specificity of the enzyme for specific recognition sites may be attributed to the loss in original steric confirmation of the histones during isolation, especially removal from DNA.
In Vitro Methylation of Histones

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