ENZYMATIC METHYLATION OF PROTEIN FRACTIONS FROM CALF THYMUS NUCLEI

Woon Ki Paik and Sangduk Kim

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

Received August 9, 1967

It was previously reported that among the various histone fractions of calf thymus only arginine-rich histone contained ε-N-methyllysine, as determined by ninhydrin color (1). Findings in our laboratory have demonstrated that arginine-rich histone contained not only ε-N-methyllysine, but also ε-N-dimethyllysine (2). On the other hand, Comb et al. (3) demonstrated that lysine-rich histone from the nuclei of Ehrlich ascites tumor cells also serves as a methyl acceptor in the crude cell-free system. We report in this paper that, with isolated calf thymus nuclei labeled with S-adenosyl-L-methionine-methyl-^{14}C, methyl-^{14}C was distributed in all the protein fractions; H_2SO_4-insoluble protein, lysine-rich histone, slightly lysine-rich histone and arginine-rich histone. However, lysine-rich histone incorporated radioactivity most actively, forming ε-N-methyllysine.

MATERIALS AND EXPERIMENTAL PROCEDURES

Materials- ε-N-Methyl-L-lysine and ε-N-dimethyl-L-lysine were prepared as described previously (4). S-Adenosyl-L-methionine-methyl-^{14}C (SAM-methyl-^{14}C; specific activity, 36.0 mC per mmole in H_2SO_4) was purchased from Nuclear Research Chemicals, Inc.

We thank Dr. M. P. Brigham for allowing us to use the Beckman automatic amino acid analyzer, and Mr. E. Pearson for skillful technical assistance with the analyzer.

Supported by NIH(AM09602-01A1 PC), NSF(B7-0280R) and NCI(CA 07174).

Methylation and fractionation of isolated calf thymus nuclei- Calf thymus nuclei were prepared by the method of Allfrey et al. (5). In order to methylate the endogenous protein, 2.3 ml of freshly isolated nuclei (103 mg protein), 1.0 ml of SAM-methyl-^{14}C (3.07×10^6 cpm), 1.2 ml of 1.0 M phosphate buffer at pH 7.2 and 0.5 ml of water were incubated for 4 hours at 37°. Fractionation of the endogenous methylated protein (Table I) was carried out according to the method of Luck et al. (6) with a slight modifications: To the incubation mixture, 0.6 ml of 2 N H_2SO_4 was added and the mixture was centrifuged at 10,000 x g for 10 minutes. The resulting precipitate was washed twice with 0.2 N H_2SO_4 to yield an H_2SO_4-insoluble fraction. To the combined supernatant and wash there was added 2.5 volumes of 95% cold ethanol and the suspension left overnight at -10°. The solution was centrifuged, and the precipitate was further washed three times with 95% cold ethanol to give an EtOH-soluble fraction. The precipitate was suspended in 10 ml of water and was further extracted three times with water to yield the sulfated histones. For the measurement of incorporated radioactivity at this stage, portions were treated to remove the acid-soluble fraction, the nucleic acids and phospholipids by the method described (5). For radioactivity counting, 10 ml of Bray's solution was used (7).

To the sulfated histones obtained by the procedure described above there was added 2.5 ml of 0.1 M $BaCl_2$. $BaSO_4$ was removed by centrifugation, and the remaining $BaCl_2$ in the supernatant as well as any possible free SAM-methyl-^{14}C was removed by dialysis. The dialyzed sample was chromatographed on a 1 x 20 cm CM-cellulose column as described by Phillips and Johns (8). The fractions corresponding to each histone were separately pooled, evaporated to dryness in vacuo, dissolved in a small amount of water, and dialyzed overnight against running water in order to remove sodium acetate. The dialyzed samples were lyophilized and hydrolyzed in 10 ml of 6 N HCl for 16 hours at 110° under reflux. The mixture was evaporated to dryness in vacuo and washed twice with water to remove HCl. A portion of the sample was analyzed with the Beckman

automatic amino acid analyzer. Elution was carried out with 0.35 M citrate buffer pH 5.84 at 28° with a flow rate of 30 ml per hour (2).

RESULTS AND DISCUSSION

Chromatogram of acid-hydrolyzate of isolated calf thymus nuclei labeled with SAM-methyl-^{14}C - When freshly isolated calf thymus nuclei were labeled with SAM-methyl-^{14}C, the nucleic acids and phospholipids removed, and the acid-hydrolyzates analyzed by means of the Beckman automatic amino acid analyzer, there appeared four radioactivity peaks in the basic region. Fig. 1 illustrates the elution pattern of some of the basic amino acids and the radioactivity pattern.

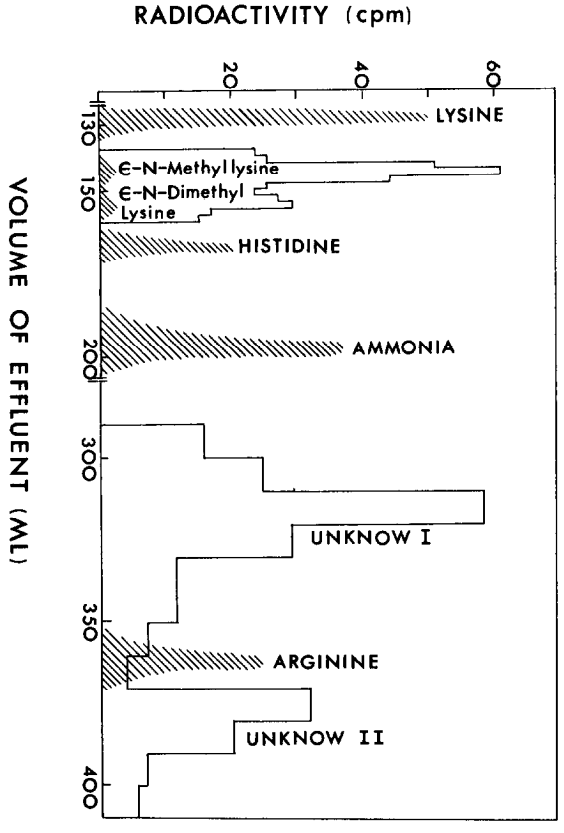

Fig. 1. Chromatogram of acid-hydrolyzate of calf thymus nuclei labeled with SAM-methyl-14-C. Ten mg protein of isolated calf thymus nuclei was used. In the figure, the peaks of ninhydrin color do not represent the actual amounts of amino acids, but rather indicate the position of the amino acids eluted. Since " basic column " was used, acidic and neutral amino acids as well as tyrosine and phenylalanine were eluted before lysine. Clear areas indicate radioactivity; shaded areas indicate positions of the amino acids.

Approximately 90% of the total radioactivity charged on the column was recovered in the four peaks in the figure. Considering the quenching effect of ninhydrin color, this represents almost complete recovery of the radioactivity. The radioactivity peaks between lysine and histidine absolutely coincided with those of ε-N-methyl-L-lysine and ε-N-dimethyl-L-lysine, respectively (2). It was previously found that commercial calf thymus arginine-rich histone contained approximately 1.7 moles of ε-N-methyllysine and 3.5 moles of ε-N-dimethyllysine per 100 moles of lysine (2). Of the remaining two peaks, unknown I was eluted before arginine and unknown II was eluted after arginine. These two radioactive peaks are rather broad in elution pattern, and it is possible that the individual peak is a mixture of more than one compound. These two radioactivity peaks could not be simple decomposition products of SAM-methyl-^{14}C, since a sample of SAM-methyl-^{14}C withstood treatment with 6 N HCl at 110° for 16 hours and all the radioactivity was recovered before lysine on the chromatogram. Methyl-^{14}C-labeled protein hydrolyzed up to 72 hours still gave rise to these two radioactive peaks. Treatment of isolated calf thymus nuclei for 3 minutes at 100° abolished completely the subsequent formation of these compounds as well as the two methylated lysine derivatives. Furthermore, the formation of these two peaks was not affected with puromycin at the concentration of 100 μg/ml. Therefore, it is obvious that these radioactive compounds are the products resulting from transmethylation between protein and SAM-methyl-^{14}C.

Fractionation of the endogenous protein - When freshly prepared calf thymus nuclei were incubated with SAM-methyl-^{14}C and the methylated endogenous protein was fractionated as described earlier, approximately half of the total incorporated radioactivity was found in the H_2SO_4-insoluble fraction, and about 40% in the various histones. Since all the fractionated proteins contained radioactivity, the question arises whether there are any qualitative

Table I

Fractionation of the endogenous protein of isolated calf thymus nuclei labeled with SAM-methyl-14-C.

Fraction	Radioactivity	Per cent
	cpm	%
H_2SO_4-insoluble	72,660	54.9
H_2SO_4-soluble:		
EtOH-soluble	1,660	1.3
Sulfated histone:		
lysine-rich histone	26,070	19.7
slightly lysine-rich histone	20,340	15.4
arginine-rich histone	7,230	5.5
H_2O-insoluble	4,300	3.3

differences in radioactivity incorporation between them.

As seen in Table II, all the proteins listed in the table have radioactivity incorporated into the four compounds. However, some quantitative differences exist between the proteins. ε-N-Methyllysine is, in general, more radioactive than ε-N-dimethyllysine at the ratio of about 2:1.

Table II

Chromatographic analysis of acid-hydrolyzates of various proteins of isolated calf thymus nuclei labeled with SAM-methyl-14-C.

Protein	Charged on column	Recovered as				
		ε-N-methyl-lysine	ε-N-dimethyl-lysine	I*	II*	Recovery
	cpm	cpm	cpm	cpm	cpm	%
H_2SO_4-insoluble	11,770	1,940	820	5,180	2,290	87
Lysine-rich histone	17,380	10,220	540	1,570	1,210	78
Slightly lysine-rich histone	10,170	530	210	6,010	1,390	80
Arginine-rich histone	3,500	490	200	1,620	670	85

* The unknown I or II.

However, in the case of lysine-rich histone, the amount of radioactivity in ε-N-methyllysine is almost 20 times higher than ε-N-dimethyllysine. In H_2SO_4-insoluble protein and arginine-rich histone, the radioactivity in the unknown I is almost twice as high as in the unknown II. In slightly lysine-rich histone more than four times radioactivity was found in the unknown I than in II, while the amounts are almost equal in lysine-rich histone. It is also

noted that only lysine-rich histone has a much larger amount of radioactivity in the methylated lysine than in the sum of unknown I and II. Since we do not have a clear knowledge of the biological function of histone and of the biochemical significance of the methylation of protein, the significance of the results in Table II is not clear at present.

It is of great interest to note that not only arginine-rich histone, but lysine-rich as well as slightly lysine-rich histones were highly methylated on the ε-amino group of the lysine residue. Since Murray (1) and we (2) found, on the basis of ninhydrin reaction, that ε-methylated lysine is present only in arginine-rich histone, the present finding suggests that lysine-rich histone and slightly lysine-rich histone, particularly the former, are metabolically very active.

The existance of radioactive methylated lysine in the H_2SO_4-insoluble protein was rather unexpected. This may be due either to " acid-unextractable " histone or to protein other than histone that may contain methylated lysine derivatives. Indeed, it was reported that the enzymatic methylation of ribosomal protein from an aquatic fungus Blastocladiella emersonii and protein from Salmonella typhimurium extract resulted in methylation only on the ε-amino group of lysine (3).

It was recently reported by Liss and Edelstein that rat skin contained an enzyme which methylated endogenous protein with SAM-methyl-^{14}C as methyl donor (9). However, the incorporated methyl-^{14}C became volatile after treatment for 20 hours at 110° in vacuum-sealed tubes using 6 N HCl. Since all four compounds described in the present paper are resistant to the above treatment, the enzyme system described by Liss and Edelstein should be different from the one discussed here.

REFERENCES

1. K. Murray, Biochemistry, 3, 10 (1964).
2. W. K. Paik and S. Kim, Biochem. Biophys. Res. Communs., 27, 479 (1967).
3. D. G. Comb, N. Sarkar, and C. J. Pinzino, J. Biol. Chem., 241, 1857 (1966)

4. L. Benoiton, Can. J. Chem., 42, 2043 (1964).
5. V. G. Allfrey, A. E. Mirsky, and S. Osawa, J. Gen. Physiol., 40, 451 (1957).
6. J. M. Luck, P. S. Rasmussen, K. Satake, and A. N. Tsvetikov, J. Biol. Chem., 233, 1407 (1958).
7. G. A. Bray, Anal. Biochem., 1, 279 (1960).
8. D. M. P. Phillips and E. W. Johns, Biochem. J., 72, 538 (1959).
9. M. Liss and L. M. Edelstein, Biochem. Biophys. Res. Communs., 26, 497 (1967).