Arch. Pharm. Res. 9(3), 157-161 (1986)

Fractionation of Enzymatically Methylated Acid-Insoluble Proteins from Thymus Nuclei

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(Received August 4, 1986)

Abstract □ Isolated calf thymus nuclei were *in vitro* methylated with S-adenosyl-L-*methyl*-^{14}C methionine, and the proteins were fractionated according to their solubilities. Histone fraction (H_2SO_4-soluble fraction) contained approximately 60% total radioactivity incorporated, while "residual protein" which was H_2SO_4-insoluble contained the remaining radioactivity. The "residual protein" was further fractionated into various acidic proteins, which contained very little of the radioactivity. However, the protein fraction eluted from DEAE-cellulose with 0.5 N NaOH contained the largest amount of radioactivity. This protein was found to be basic in nature by amino analysis.

Keywords □ Methylated acid-insoluble proteins, Acidic proteins, Isolated calf thymus nuclei, Histones

When isolated rat liver calf thymus 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 (1, 2). 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; S-Adenosyl-L-methionine:protein-arginine N-methyltransferase; EC 2.1.1.23), while methylated lysines are the major methylated amino acids in the histones (product of protein metylase III; S-Adenosyl-L-methionine: protein-lysine N-methyltransferase; EC 2.1.1.42). We report in this paper that the H_2SO_4-insoluble protein was further fractionated into several subfractions, two of which contained almost all of the radioactivity. Amino acid analysis revealed that one of these fractions is highly basic in nature and the other is acidic.

EXPERIMENTAL METHODS

Materials

S-Adenosyl-L-(*methyl*-^{14}C) methionine (specific activity, 59 mCi/mmol) was purchased from Amersham, Arlington Heights, IL. All the reagents used were obtained from various commercial sources and were of the highest purity available.

In vitro methylation of isolated calf thymus nuclei

Calf thymus nuclei prepared by the method of Allfrey *et al.* (3) was enzymatically methylated as described previously (1, 2). The reaction mixture was incubated for 3 hours at 37℃. After incubation, 0.6ml of 2 N H_2SO_4 per 5ml of incubation mixture was added, and the mixture was centrifuged at 39,000×*g* for 30 minutes. The precipitate was broken into small pieces in order to facilitate the following extraction. Eight m*l* of 0.2 N H_2SO_4 was added to the precipitate and was left for 10 minutes before centrifugation at 39,000×*g* 10 minutes. This extraction step was repeated twice with 5ml each of 0.2 N H_2SO_4. All the supernatants were combined (H_2SO_4-soluble fraction). Over 95% of the incorporated radioactivity in this fraction was found in various histones (1). The precipitate obtained after removal of H_2SO_4-soluble fraction was now treated fractionate acidic proteins by the method of Wang (4) as follows; the precipitate was homogenized in 5ml of 0.05M Tris-HCl buffer at pH8.5,

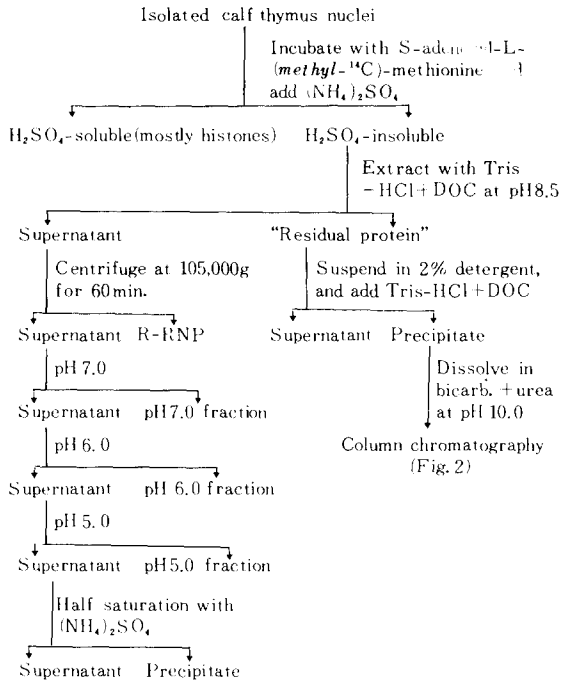

Fig.1. Flow Sheet of fractionation of methylated proteins from isolated calf thymus nuclei.

containing 0.005M $MgCl_2$. Most of the precipitate did not go into solution. Five ml of 2% deoxycholic acid (DOC) was added while stirring. The suspension was centrifuged at 39,000×*g* for 10 minutes, and the precipitate was again dissolved in 2.5ml of the above buffer and 2.5ml of DOC. It was centrifuged for 10 minutes at the above speed. The precipitate was designated as "residual protein", and further fractionation of this fraction will be described later in this paper. The combined supernatnat and washing was centrifuged at 105,000×*g* for 60 minutes, and the pellet was dissolved in the above Tris-HCl buffer (residual ribonucleoprotein or R-RNP). The pH of the supernatant was adjusted to 7.0 with 1 N acetic acid and was centrifuged at 39,000×*g* for 10 minutes. The precipitate was resuspended in 2ml of the Tris-HCl buffer (pH7.0 fraction). The pH of the supernatant was adjusted to 6.0 and centrifuged at 39,000×*g* for 10 minutes. The precipitate was dissolved in 15ml of the above buffer (pH6.0 fraction). The pH of the supernatant was brought to 5.0 and the precipitate at 39,000×*g* for 10 minutes was dissolved in 5.4ml of the buffer (pH5.0 fraction). The supernatant was further treated with an equal volume of saturated ammonium fulfate (at room temperature), and the precipitate at 39,000×*g* for 10 minutes was suspended in 2.5ml of the buffer. This method was originally developed to fractionate nuclear acidic proteins of rat liver. For easier understanding of this procedure, a flow sheet is prepared in Fig.1.

The "residual protein" obtained above was extremely difficult even to suspend. After several trials, it was found that common laboratory detergent is most efficient to suspend it. Therefore, the "residual protein" was suspended in 2% detergent solution by aid of glass homogenizer (detergent; Cat. No. 4-320, Bio-degradable Sparkleen By Fisher Scientific Co.). The final volume was brought to 20ml. Equal volumes of the above Tris-HCl buffer and DOC were added into suspension, and the suspension was centrifuged at 39,000×*g* for 10 minutes. The supernatant was discarded. The precipitate was then dissolved in 100ml of 0.02M bicarbonate-carbonate buffer at pH10.0, containing 0.5M urea.

Ten ml of this suspension was charged on DEAE-cellulose column (1×15cm) which had previously been equilibrated with the above bicarbonate buffer, and the column was successively eluted with 1.0M bicarbonate-carbonate buffer at pH10.0 containing 5M urea, 0.5N NaOH and 1.0N NaOH (Fig.2).

Proteins were hydrolyzed in 6N HCl in vacuum-sealed tube at 110℃ for 20 hours. In the case of proteins eluted in 0.5N or 1.0N NaOH, the proteins were dialyzed against water prior to acid-hydrolysis. The mixture was evaporated to dryness *in vacuo* and washed twice with water to

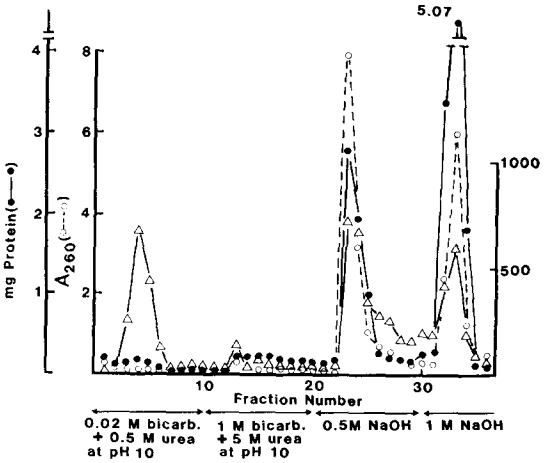

Fig.2. Chromatographic separation of the "residual protein". Detailed experimental procedures are described in text.

Table I. Distribution of various proteins and radioactivity in calf thymus nuclei.

Fractions	Protein				Radioactivity*		
	Volume (*ml*)	mg/*ml*	Total (mg)	Percent	Total cpm	cpm/mg protein	Percent
H_2SO_4-soluble	24.0	2.86	68.6	45.8	41,664	606	59.4
"Residual protein"	12.5	5.72	71.5	47.8	27,588	386	39.4
R-RNP	2.5	0.50	1.3	0.9	225	173	0.3
pH 7 fraction	2.0	0.14	0.3	0.2	0	0	0
pH 6 fraction	15.0	0.42	6.3	4.2	525	83	0.7
pH 5 fraction	5.4	0.28	1.5	1.0	64	43	0.1
Precipitate by $(NH_4)_2SO_4$	2.5	0.09	0.2	0.1	0	0	0

*The fractions were treated to remove nucleic acids and phospholipids by the method of Allfrey et al. (3).

remove HCl. A portion of the sample was analyzed for its amino acid composition with the Beckman automatic amino acid analyzer. For radioactivity counting, 10ml of scintillation solution was used. Protein concentration was measured by the method of Lowry *et al.* (5), DNA by the use of diphenylamine (6), and RNA by the method of Dische and Schwartz (7).

RESULTS

Fractionation of nuclear proteins methylated in vitro

Table I lists the results on the distribution of radioactivity and proteins in various fractions of calf thymus nuclei *in vitro* with S-adenosyl-L-(*methyl*-^{14}C) methionine. Since 145.8mg of protein was processed, the overall recovery of the protein was calculated to be 104%. These results show that approximately 50% of the total proteins was recovered in "residual proteins" and 45% in acid-soluble fraction (mostly histones). Among the various acidic proteins, pH6.0 fraction contained the largest amount of protein. Since isolated calf thymus nuclei became aggregated during incubation with S-adenosyl-L-(*methyl*-^{14}C)-methionine in phosphate buffer, it is possible that the distribution pattern of proteins listed in Table I might be due to aggregation which hinders a smooth fractionation. However, control fractionation without incubation with S-adenosyl-L-(*methyl*-^{14}C) methionine gave almost identical results. Table I also shows that all the radioactivity after removal of H_2SO_4-soluble fraction was found in the "residual proteins". "Acidic protein" (4) do not contain any significant amount of radioactivity, even though fractions constitute about 7% of the total protein.

Since "residual proteins" contained all the radioactivity after H_2SO_4-soluble fraction, was further fractionated on DEAE-cellulose column chromatography. As shown in Fig.2, radioactivity was separated into three peaks. Large amount of protein was recovered in the fractions eluted with 0.5N and 1.0N NaOH, particularly in the latter. These two peaks contained DNA as well as RNA, in varying amounts depending on the preparations. Therefore, definite ratio of DNA:RAN:protein is rather meaningless under the present condition. It is seen in Fig.2 that specific radioactivity and total amount of radioactivity in the protein peak which was eluted with 0.5N NaOH was far greater than those in the 1.0N NaOH-eluted peak.

Table II lists the amino acid composition of various protein fractions from calf thymus nuclei fractionated as described in Fig.1. The amino acid composition of the H_2SO_4-soluble fraction agrees well with the value of unfractionated calf thymus histone, averaged from several published date (8). The composition of the pH6.0 fraction is quite different from that of rat liver fraction (4). From the ratio of acidic amino acid to basic amino acids, it can be seen that the protein eluted with 0.5N NaOH has basic characteristics. However, this protein seems to be somewhat different in composition than the histones. The protein eluted with 1.0N NaOH is not as acidic as the pH6.0 fraction. It is quite possible that this protein may have been contaminated with basic protein.

DISCUSSION

It is now well established that protein methylation is carried out by a variety of protein meth-

Table II. Amino acid composition of various nuclear protein fractions of calf thymus nuclei.

Amino acid*	Unfractionated histone		pH 6.0 fraction		Fraction eluted with NaOH	
	Found	Literature**	Found	Literature***	0.5N	1.0N
Alanine	13.8	13.2	9.6	10.2	9.4	7.9
Arginine	6.9	8.3	8.9	5.4	8.3	6.8
Aspartic acid	4.6	5.1	10.0	7.0	9.8	8.3
1/2 Cystine				0.6	0	
Glutamic acid	8.8	8.8	10.3	8.0	1.4	12.3
Glycine	9.9	8.4	10.1	7.9	9.4	9.6
Histidine	1.8	1.9	2.6	1.9	3.6	4.0
Isoleucine	4.3	4.3	4.3	4.8	5.2	4.3
Leucine	7.3	7.6	9.3	10.3	9.9	8.7
Lysine	13.2	14.6	2.1	7.0	9.4	8.2
Methionine	–	0.9	trace	3.0	1.2	trace
Phenylalanine	1.6	2.0	4.0	5.2	4.3	3.2
Proline	4.2	5.1	5.3	6.4	4.4	3.6
Serine	6.1	5.8	7.8	5.2	6.9	6.3
Threonine	6.9	6.0	6.2	5.4	6.5	5.3
Tyrosine	1.8	2.2	2.9	4.0	3.1	3.0
Valine	8.9	6.3	6.5	6.5	7.0	8.7
Asp+Glu/Lys+His+Arg	0.61	0.56	1.49	1.05	0.53	1.08

*The values are percentage of amino acid recovered.
**Average value from several published data (8)
***Reference 4.

yltransferases (9, 10). Protein methylase I (S-Adenosyl-L-methionine:protein-arginine N-methyltransferase; EC 2.1.1.23) methylates the guanidino group of arginine residues; protein methylase II (S-Adenosyl-L-methionine:protein-carboxyl O-methyltransferase; EC 2.1.1.24) methylesterifies free carboxyl groups of glutamyl and aspartyl residues, and protein methylase III (S-Adenosyl-L-methionine:protein-lysine N-methyltransferase; EC 2.1.1.43) methylates the ε-amino group of lysine residues. These enzymes have been shown to be highly specific towards the amino acid residues involved. Additionally, they are highly specific towards the protein substrate species. Thus, there are two protein methylase I's thus far identified, one of which is active with only histone while the other with myelin basic protein. For this reason, we attempted to identify the nonhistone nuclear protein (s) which is methylated *in vitro*. However, we have not been successful in resolving the nonhistone nuclear proteins, mainly due to their insolubilities.

HMG-1 and HMG-2 proteins are nonhistone chromosomal proteins, known to contain high proportions of N^G,N^G-dimethylarginine (11, 12). Therefore, it is highly likely that the N^G-methylarginine containing proteins eluted from the DEAE-cellulose shown in Fig.2 are part of the HMG nonhistone class. Further studies in the resolution of these HMG proteins from the eluates described in this paper remain to be explored.

Methylation of nonhistone nuclear proteins has also been described by Liew and Suria (13). In these experiments, rats were injected intraperitoneally with S-adenosyl-L-(*methyl*-^{3}H) methionine. Upon isolation of the kidney, heart and liver, it was found that in all these organs the incorporation of radioactivity was higher in the nonhistone chromosomal protein fraction than the histone fraction. Also among the organs tested, the kidney nonhistone protein fraction was labeled to the greatest extent. On further analysis using isoelectro-focusing polyacrylamide gel electrophoresis, two fractions of acidic proteins, which focused at pH

6.2 and 6.5, were most highly methylated. However, they did not attempt to identify the nature of amino acid (s) involved.

Finally, in spite of extensive research efforts by us and other research groups, the biological significance of protein methylation remains clouded. However, it has been observed that histone-specific protein methylase I and III activities were elevated whenever cell proliferation was accelerated, such as in fast-growing Morris and Novikoff hepatomas (2), in fetal tissues (14), continuously dividing HeLa S-3 cell culture (15), and during hepatic regeneration of adult rat liver (16).

ACKNOWLEDGEMENTS

This work was supported by Research Grants AM09602 from the National Institute of Arthritis, Diabetes, Digestive and Kindeney Diseases, CA12226 and CA12227 from the National Cancer Institute, and RG1765-A-1 from the National Mutiple Sclerosis Society.

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