NON-ENZYMATIC METHYLATION OF PROTEINS WITH S-ADENOSYL-L-METHIONINE

Woon Ki PAIK, Hyang Woo LEE and Sangduk KIM
Fels Research Institute and Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pa. 19140, U.S.A.

Received 10 August 1975

1. Introduction

It is now well established that various side chains of some proteins are methylated in vivo [1,2]. These protein methylation reactions are highly specific. Thus, protein methylase I (S-adenosylmethionine: protein–arginine methyltransferase; EC 2.1.123) methylates the guanidino group of arginine residues [3], protein methylase II (S-adenosylmethionine: protein–carboxyl methyltransferase; EC 2.1.1.24) methylates free carboxyl group of aspartyl and glutamyl residues [4], and protein methylase III (S-adenosylmethionine: protein–lysine methyltransferase; EC 2.1.1.25) methylates the ϵ-amino group of lysine residues. We report here some experimental results which indicate that these enzyme-catalyzed protein methylation reactions can occur in the absence of enzyme with S-adenosyl-L-methionine as methyl donor.

2. Materials and methods

S-Adenosyl-L-[methyl-^{14}C] methionine (spec. act. 58 mCi/mmol.) was obtained from New England Nuclear Corporation. All the proteins used were purchased from Sigma Chemical Co., and the rest of reagents from various local sources.

Experimental procedures are similar to those for the enzyme-catalyzed reactions [3–5], except enzymes are omitted from the reaction mixture. Assay method for protein methylation consists of determination of amount of radioactivity remaining in the 15% trichloracetic acid-insoluble precipitate after reacting proteins with S-adenosyl-[methyl-^{14}C]-methionine. Routinely otherwise specified, the incubation mixture contained the following: 0.1 ml of protein suspension (3 mg in water), 0.1 ml of buffer (0.25 M citrate-phosphate buffer at pH 6.3 or 0.5 M sodium bicarbonate buffer at pH 12.5), 0.1 ml of S-adenosyl-L-[methyl-^{14}C] methionine (4.98 nmmol; 105 cpm/pmol) and 0.2 ml of water. After 5 or 20 min of incubation at 37°C, the reaction was terminated by the addition of 0.5 ml of 30% trichloracetic acid. It was found that initial rate of reaction was linear up to about 30 min. The incubation mixture was treated to remove any possible contamination of nucleic acid and phospholipids by treating it at 90°C for 15 min with 15% trichloracetic acid followed by ethanol extraction at 70°C for 15 min. The sample was then transferred to a scintillation vial which contained 10 ml of scintillation solution (Aquasol, product of New England Nuclear Corporation). The blank was prepared by adding protein suspension after the addition of trichloracetic acid, and assay value was corrected for the blank.

3. Results and discussion

3.1. *pH-Relationship*

When egg white globulin was incubated with S-adenosyl-L-[methyl-^{14}C] methionine in various pH's, two pH optima are found (fig.1); one at 6.3 and the other at 12.5. Furthermore, various buffers at a given pH have a minimal effect on the incorporation of radioactivity to trichloracetic acid-insoluble precipitate.

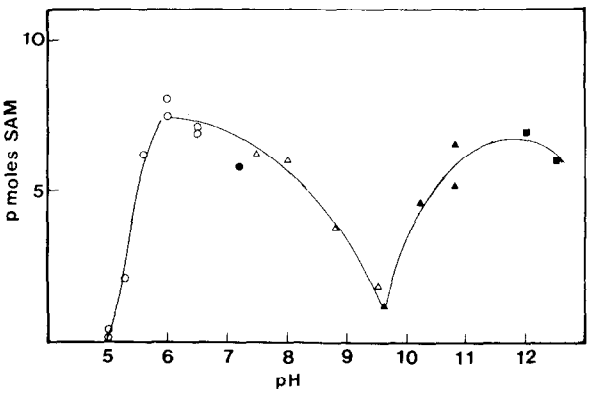

Fig.1. pH-Curve. Various buffers at the final concentration of 0.1 M were used. Incubation time was 5 min. ○——○ represents citrate-phosphate, ●——● for phosphate, △——△ Tris–HCl, ▲——▲ sodium bicarbonate and ×——× for glycine-NaOH buffer. The rest of experimental procedures are described under Materials and methods.

3.2. *Specificity*

Table 1 lists the results on the specificity of the reaction for proteins. At pH 6.3, egg white globulin incorporated the radioactivity most actively, followed by *Bacterium cadaveris* (acetone powder), lysozyme and oxidized ribonuclease in decreasing order. On the other hand, most of the commercially available pure proteins were good acceptors of the radioactivity of S-adenosyl-L-[methyl-^{14}C]methionine at pH 12.5, with polyarginine being the most active. Heating the egg white globulin at 100°C for 5 min abolished completely the radioactivity-accepting activity at pH 6.3 whereas the activity at pH 12.5 was decreased by 50%. This indicates that the tertiary structure of the protein plays a crucial role in in accepting the radioactivity from S-adenosyl-L-[methyl-^{14}C]methionine at pH 6.3.

S-Adenosyl-L-methionine is stable in acidic solution, but not in alkaline pH [6]. However, the

Table 1
Specificity of non-enzymatic methylation of various proteins by S-adenosyl-L-methionine

Proteins	Rate of non-enzymatic methylation (%)	
	at pH 6.3	at pH 12.5
Egg white globulin	100.0[a]	100.0[a]
Bacterium cadaveris (acetone powder)	6.0	22.0
Lysozyme	3.2	169.4
Ribonuclease (oxidized)	1.0	113.0
Ribonuclease (reduced)	0	141.8
Histone type II-A (Sigma)	0	117.5
Polyarginine	0	662.5
Polylysine	0	119.8
Trypsin inhibitor	0	24.8
Urease	0	23.4
Bovine serum albumin	0	23.3
Trypsin	0	6.7
Polyalanine	0	0

[a]100% of rate of non-enzymatic at pH 6.3 represents 48.36 pmol of S-adenosyl-L-[methyl-^{14}C]methionine used per 20 min, and 100% at pH 12.5 represents 17.72 pmol of S-adenosyl-L-[methyl-^{14}C]methionine per 5 min. The rest of experimental procedures is described under Materials and methods.

S-adenosyl-L-[methyl-^{14}C] methionine purified by Dowex-50 H^+ resin [7] was found as effective as the original commercially obtained sample. Furthermore, when 16 000 cpm of S-adenosyl-L-[methyl-^{14}C]-methionine was incubated with 9 mg of egg white globulin at pH 6.3 (relatively a large amount of protein to S-adenosyl-L-methionine), approximately 30% of the total radioactivity became trichloracetic acid-insoluble in 4 h. These results strongly indicate that a contaminant in S-adenosyl-L-[methyl-^{14}C-]-methionine is not the contributing factor for this reaction.

3.3. *Stability of the reaction product*

Before attempting to elucidate the identity of the reaction product, we have examined the stability of the incorporated radioactivity. Egg white globulin was first labeled with S-adenosyl-L-[methyl-^{14}C]-methionine at pH 6.3, treated to remove trichloracetic acid-soluble radioactivity, nucleic acids and phospholipids, the sample was dialyzed against water at cold room for 3 h, and aliquots were introduced to solutions of varying pH's. It is seen in fig.2 that the incorporated radioactivity is highly stable at pH's below 5, and becomes increasingly unstable as the pH of the solution increases. This characteristic of the reaction product is quite analogous to those of the product of protein methylase II (S-adenosyl-methionine: protein–carboxyl methyltransferase) [4].

Unlike to the radioactivity incorporated at pH 6.3, the radioactivity incorporated at pH 12.5 did not become trichloracetic acid-soluble in any pH at room temperature. However, the radioactivity became volatile upon hydrolysis of the protein at 110°C in 6 N HCl.

3.4. *Identification of the product*

As illustrated in fig.2, the radioactivity incorporated at pH 6.3 is highly unstable in alkaline pH's (becomes trichloracetic acid-soluble). Therefore, when egg white globulin which was labeled with S-adenosyl-L-[methyl-^{14}C] methionine at pH 6.3 was resuspended in pH 7.8 phosphate buffer and the mixture was distilled according to the published method [8], over 50% of the originally incorporated radioactivity became distillable. When this distilled radioactive material was reacted with 3,5-dinitrobenzoyl chloride [4,9], all of the radioactivity was found in the methyl ester of 3,5-dinitrobenzoate. Again, this characteristic is very much analogous to that of protein methylase II reaction [4]; in both non-enzymatic and enzymatic reactions, the optimum pH for the reactions are around 6, and [methyl-^{14}C]-methyl alcohol was liberated in alkaline pH solution. Furthermore, the activation energy of the non-enzymatic reaction was found to be about 21 kcal/mol which is identical with that found in the enzymatic

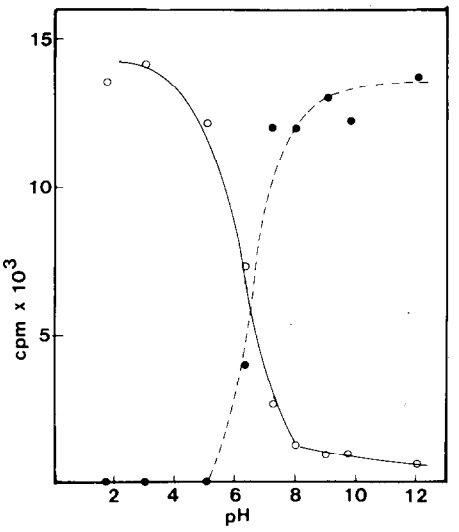

Fig.2. Stability of the incorporated radioactivity. 13.5 ml of egg white globulin (500 mg), 5.0 ml of 0.25 M citrate-phosphate buffer at pH 6.3 and 7.5 ml of S-adenosyl-L-[methyl-^{14}C] methionine (3.74 × 10^7 cpm) were incubated at 37°C for 4 h. After removing trichloracetic acid-soluble, nucleic acids and phospholipids, the sample was dialyzed against water in the cold room for 3 h. A 2.0 ml of aliquot was introduced into 0.5 ml of different buffer solutions and 0.5 ml of absolute alcohol in order to prevent growth of bacteria. The tubes were left at room temperature for 24 h. Trichloracetic acid was added at the final concentration of 15%, the samples were centrifuged for 10 min in clinical centrifuge (table-top type). The radioactivity in the soluble and insoluble fraction was determined. ○——○ represents the cpms remaining in the trichloracetic acid-insoluble precipitate and ●——● the cpms in the soluble fraction. pH 1.7 solution was made with 0.1 N HCl; pH 3 by 0.5 M glycine-HCl; pH 5.0 with 0.2 M citrate; pH 6.3 with 0.25 M citrate-phosphate; pH 7.2 and 8.0 with 0.5 M phosphate; pH 9.0 with 0.5 M Tris–HCl; pH 9.74 with 1.0 M ammonium-ammonium chloride; pH 12.0 with 0.1 N NaOH.

reaction [4]. It is therefore concluded that in both reactions S-adenosyl-L-methionine esterified free carboxyl residues of the protein molecule. No further attempts were made to identify the product formed at pH 12.5.

The reaction described in this communication is non-enzymatic. The possibility that egg white globulin contains protein methylase II activity is extremely unlikely; protein methylase II is extremely unstable, particularly in the absence of protective agents such as glycerol [10] and protein methylase II activity was not detected in fresh egg white (exogenously added substrate such as histone did not increase the amount of radioactivity incorporated over the value in the absence of the added substrate).

Histones have been demonstrated to be acetylated non-enzymatically with acetyl CoA [11], acetyl phosphate or acetyl adenylate [12]. However, non-enzymatic methylation of proteins with S-adenosyl-L-methionine has never been demonstrated until now. The non-enzymatic methylation at pH 6.3 appears to be highly specific for the proteins involved (table 1). Our preliminary experiments with developing chicken egg indicate that the degree of non-enzymatic methylation of egg white decreases sharply in the first 4 days of hatching (decreased by approx. 80%). The biological significance of this decrease is not clear at present.

Acknowledgements

This work has been supported by research grants AM 09602 from the National Institute of Arthritis and Metabolic Diseases, CA 10439 and CA 12226 from the National Cancer Institute, and GM 20594 from the National Institute of General Medical Sciences.

References

[1] Paik, W. K. and Kim, S. (1971) Science, 174, 114.
[2] Paik, W. K. and Kim, S. (1975) in: Advances in Enzymology (Maister, A., ed.) Vol. 42, p. 227, John Wiley & Sons.
[3] Paik, W. K. and Kim, S. (1968) J. Biol. Chem., 243, 2108.
[4] Kim, S. and Paik, W. K. (1970) J. Biol. Chem., 245, 1806.
[5] Paik, W. K. and Kim, S. (1970) J. Biol. Chem., 245, 6010.
[6] Schlenk, F. (1965) in: Transmethylation and Methionine Biosynthesis (Shapiro, S. K. and Schlenk, F., eds) p. 53, University of Chicago Press.
[7] Schlenk, F. and DePalma, R. E. (1957) J. Biol. Chem., 229, 1037.
[8] Kim, S. and Paik, W. K. (1971), Anal, Biochem., 42, 255.
[9] Neish, A. C. (1957) in: Methods in Enzymology Colowick, S. P. and Kaplan, N. O., eds) Vol. 3 p. 255, Academic Press, New York.
[10] Kim, S. (1973) Arch. Biochem. Biophys., 157,476.
[11] Paik, W. K., Pearson, D. B., Lee, H. W. and Kim, S. (1970) Biochim. Biophys. Acta, 245, 513.
[12] Ramponi, G., Manao, G. and Camici, G. (1975) Biochemistry, 14, 2681.