SELECTIVE METHYLATION OF ROUS SARCOMA VIRUS GLYCOPROTEINS BY PROTEIN METHYLASE II

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1. Introduction

Protein methylase II (*S*-adenosyl-L-methionine: protein carboxyl-*O*-methyl-transferase, EC 2.1.1.24) catalyses the transfer of the methyl group from *S*-adenosyl-L-methionine (AdoMet) to free carboxyl groups (Asp and/or Glu) of a protein [1]. The enzyme thus neutralizes negatively charged free carboxyl residues by the production of methyl esters, which are chemically unstable and are hydrolyzed enzymatically [2] or spontaneously [3] at neutral or alkaline pH, yielding methanol. Although the enzyme is widely distributed in prokaryotic and eukaryotic cells, its role is well defined only in the case of bacterial chemotaxis [4,5]. Methyl-accepting proteins have been found in various tissues such as adrenal medulla [6], leukocytes [7], red blood cells [8], blood platelets [9], parotid [10] and pituitary [11] glands as well as in mammalian spermatozoa [12]. We describe here the enzymatic methylation of five different enveloped viruses: Rous sarcoma virus (RSV), Friend virus, mouse sarcoma virus (MSV), mouse leukemia virus (MLV) and mouse mammary tumor virus (MMTV) in vitro by a purified protein methylase II from chick embryos. Among the seven purified viral proteins of RSV only the two external glycoproteins gp 80 and gp 35 can be methylated in vitro.

2. Material and methods

Growth of chick embryo fibroblasts (CEF), preparation of cell-free extracts, production and purification of RSV SR_4 were as described earlier [21]. Radioactive RSV was produced by adding 0.25 μCi/ml of a mixture of ^{14}C-labelled amino acids (1.75 mCi/mg amino acids) to a culture of RSV-transformed CEF. The medium used was Eagle's MEM with 2% of newborn calf serum, and the amino acid concentration was lowered by 90% during labelling.

Gel filtration of viral proteins: a purified [^{14}C]-labelled virus pellet (10 mg of proteins corresponding to 260 000 cpm) was solubilized in 1.5 ml of 8 M guanidine hydrochloride (GuHCl) containing 2% β-mercaptoethanol and 50 mM Tris–HCl pH 8.5 for 1 h at 60°C. Chromatography was performed as described by Fleissner [18] on a bio-gel A 5 m column equilibrated with 6 M GuHCl containing 1% β-mercaptoethanol and 50 mM sodium phosphate buffer pH 6.5. Fractions of 500 μl were collected and 25 μl of each fraction counted. The pooled fractions corresponding to the viral proteins were dialyzed first against a small volume of 2.5 mM sodium phosphate buffer pH 7.5 containing 1% β-mercaptoethanol, then overnight against increasing volumes of the same buffer containing 10 mM β-mercaptoethanol. Under these conditions important precipitation of proteins was avoided. The fractions were then centrifuged at 8000 × *g* for 5 min, freeze-dried and the residues dissolved in distilled water containing 10 mM β-mercaptoethanol. The fractions were centrifuged again, and the proteins [22] and the radioactivity determined in the supernatant which contained the viral proteins.

PM II was purified from 45 (11-day-old) chick embryos (2.5 g of proteins). After homogenizing in 5 mM sodium borate buffer pH 9.3 containing 1 mM EDTA, and 2.5 mM β-mercaptoethanol, the material was centrifuged at 100 000 × *g* for 1 h. The supernatant was brought to 25% saturation with $(NH_4)_2SO_4$ at 0°C after 3 h, the precipitate was removed and the supernatant brought to 60% saturation.

The precipitate formed in 3 h was suspended in

5 mM sodium phosphate buffer pH 8.0 containing 1 mM EDTA and 2.5 mM β-mercaptoethanol and applied to a Sephadex G-100 column (90 × 4.5 cm) equilibrated in the same buffer. The active fractions were pooled, freeze-dried, and treated batchwise with DEAE Sephadex A50 in borate buffer [14]. The four washings with 100 mM NaCl in the same buffer were then freeze-dried and dialyzed against sodium phosphate buffer containing 40% glycerol. The enzyme was purified 110-fold with an overall yield of 17%. PM II was assayed by the method of Kim and Paik [1]: 100 μg of γ-globulins (Sigma) or 50 μg of purified RSV, 10 μl of 0.05 M citrate buffer pH 6.0, 2.5 μCi of [^{3}H]AdoMet (5 Ci/mmol, The Radiochemical Centre, Amersham) or 2.5 μCi of [^{14}C]AdoMet (50 mCi/mmol, Commissariat à l'Energie Atomique, France), 60 μg of proteins of a 12 000 × *g* supernatant from a CEF crude extract or 15 units of purified PM II in a total volume of 100 μl were incubated for 30 min at 37°C. The reaction was stopped by adding 1 ml of water, immediately followed by 1 ml of 50% trichloroacetic acid (TCA). The precipitate was recovered on GF/C filters as described earlier [20].

3. Results and discussion

As shown in table 1 RSV particles are as good a substrate as γ-globulins for CEF protein methylase II (PM II). No PM II activity was detected when RSV was used as enzyme source. Furthermore RSV could not be methylated by the two other methylases, PM I (*S*-adenosyl methionine: protein arginine methyltransferase EC 2.1.1.23) and PM III (*S*-adenosyl methionine: protein lysine methyltransferase EC 2.1.1.25). Methylation of the virus by PM II was inhibited by *S*-adenosyl-L-homocysteine (AdoHcy), the natural inhibitor of all methyl transferases [13] and no stimulation was observed when the activity was measured in the presence of a nonionic detergent which disrupts viral particles. This result suggests that methylated proteins are external, thus accessible to the enzyme in the intact particle.

In order to have a larger amount of enzyme, PM II was partially purified from chick embryos by a standard procedure [14]. In this enzyme preparation, purified 110-fold, the endogenous methylation was reduced to about 10%. The kinetic constants were as follows: K_m AdoMet 1.5 μM; K_i AdoHcy 0.7 μM; K_i Sinefungin [15] 0.9 μM; compound 9145 [15] 0.08 μM; and SIBA [16] >6000 μM. Purified RSV and four other enveloped oncogenic RNA viruses could be methylated in vitro by this enzyme. The specific activities expressed as pmol methyl groups incorporated/mg of virus were: MSV 142; MLV 166; MMTV 94; Friend virus 222.

Table 1
Methylation of γ-globulins and RSV by a crude CEF extract

Substrate	−AdoHcy cpm	+AdoHcy cpm
γ-globulins 100 μg	1580	245
No substrate	673	199
RSV 50 μg	1244	285
RSV without CEF extract	62	45
RSV + 0.2% triton X-100	1325	

20 μl (60 μg proteins) of a 12 000 × *g* supernatant from a CEF crude extract were used as enzyme source and incubated with 2.5 μCi of [^{3}H]AdoMet and substrates as indicated (concentration of AdoHcy: 100 μM). Results are expressed in cpm

The carboxyl methyl esters formed enzymatically are known to be unstable at neutral or alkaline pH [3]. The high instability of the RSV methyl ester is shown in table 2. 50% of the methyl esters were hydrolyzed in 1 h at pH 6 at 20°C and 95% at pH 9 under the same conditions. In 10% TCA and at pH 3 the ester is quite stable.

RSV contains 9 major polypeptide components [17]. Two external glycoproteins: gp 80 and gp 35, five structural proteins: p 27, p 19, p 15, p 12, p 10 and the two subunits of reverse transcriptase p 92 (β) and p 62 (α).

To determine whether the methyl acceptor proteins of RSV are glycoproteins, particles labelled with a mixture of [^{14}C]amino acids were disrupted by 8 M GuHCl at 70°C and the viral proteins purified by gel filtration in the presence of 6 M GuHCl [18] (fig.1). Each of the seven fractions were dialyzed and freeze-dried. The fractions were then tested in vitro as subtrates of partially purified PM II. As expected, the main methyl acceptor proteins were the two envelope glycoproteins, both with a specific activity of 1400 pmol/of [^{14}C]methyl group incorporated/mg glycoprotein in 30 min at 37°C (fig.2a). This activity is seven-fold higher than the activity measured with the whole particles. Fig.2b shows that gp 80 is the

Table 2
Stability of the viral carboxymethyl ester

Incubation time (h)	10% TCA	Percentage of residual activity (pH)			
		3.0	4.5	6.0	9.0
1	98	95	62	51	5
2	97	91	55	38	4
3	97	86	51	37	2
4	89	68	47	27	1
20	89	72	47	27	1

Purified RSV (750 μg viral protein) was enzymatically methylated with 10 μCi of [^{14}C]AdoMet (50 mCi/mmol, Commissariat à l'Energie Atomique, Saclay, France) and 15 units of purified PM II (1 unit catalyzes the transfer of 1 pmol of [^{14}C]-CH_3 in 30 min) in a total volume of 1.1 ml. The reaction was stopped by 1 mM AdoHcy and the mixture divided in four parts. Each part was incubated simultaneously in the following buffer solutions at 20°C: 0.25 M citrate pH 3.0 and 4.5, 0.25 M citrate-phosphate pH 6.0, 0.25 M Tris–HCl pH 9.0, and 10% TCA. At the indicated times, of each mixture 25 μl were taken, precipitated with 50% TCA and the radioactivity counted. Results are expressed in percentage of remaining radioactivity (100% radioactivity represents 750 cpm)

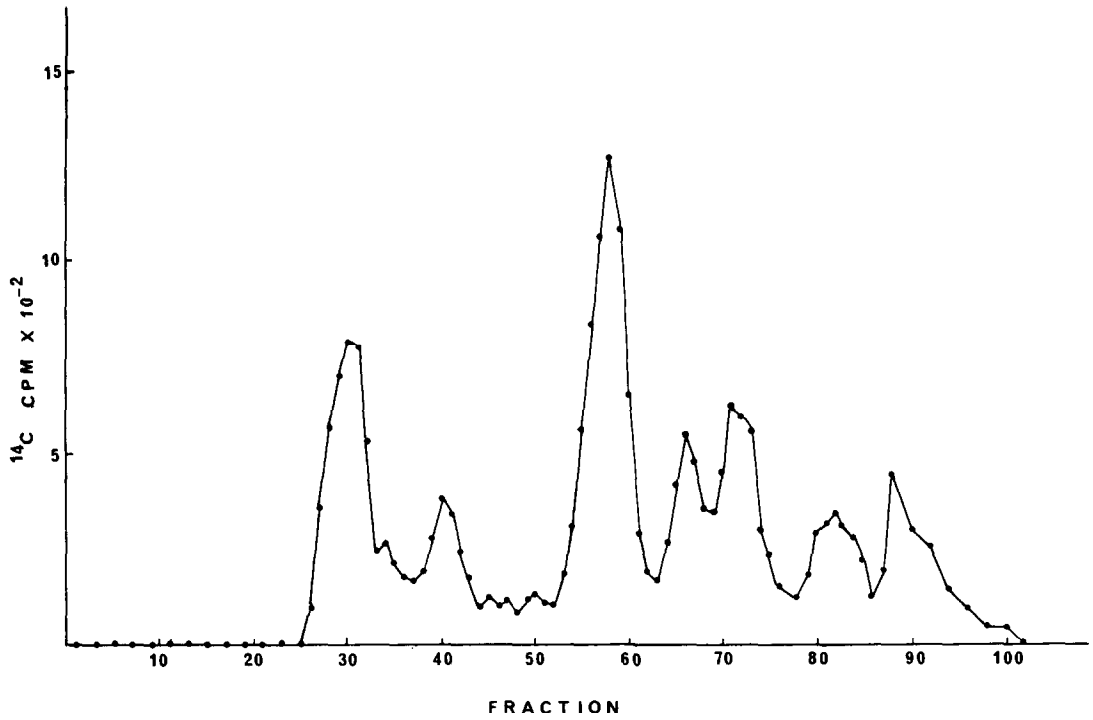
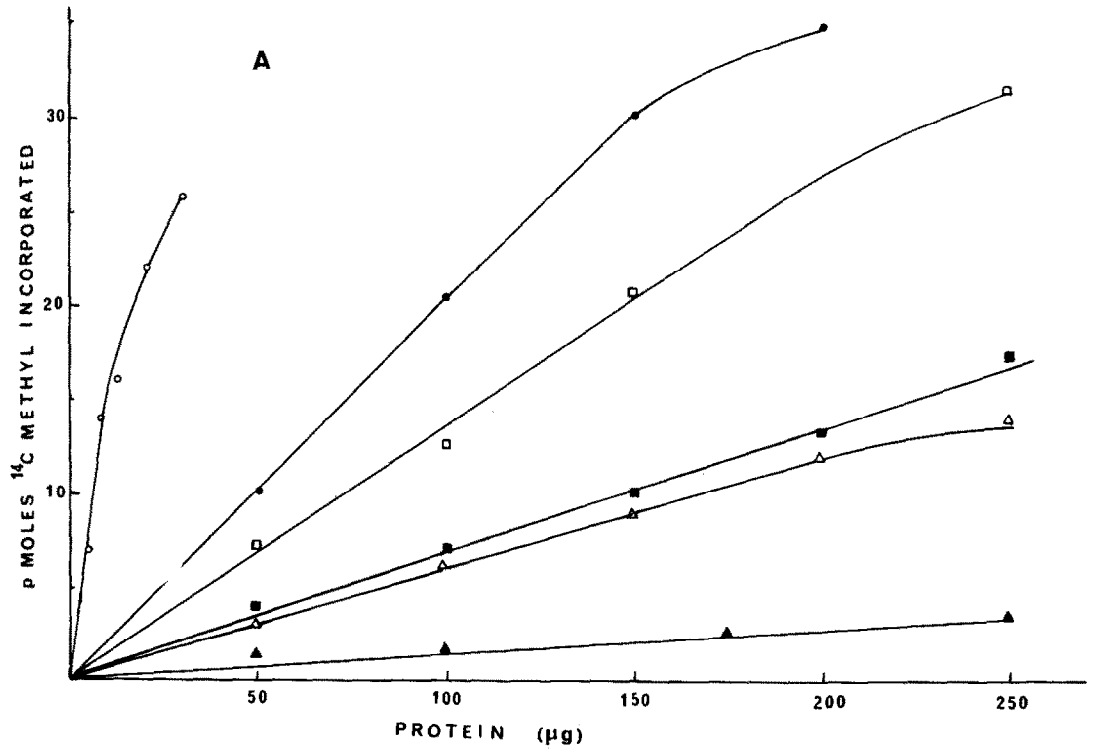


Fig.1. Gel filtration of [^{14}C]RSV proteins in the presence of 6 M GuHCl. Fractions No. 24–36 correspond to gp 35, No. 37–44 to gp 80, No. 52–62, No. 66–75 to p 19 + p 15, No. 78–87 to p 12 and No. 88–97 to p 10. (For experimental details see section 2).


A
p MOLES ^{14}C METHYL INCORPORATED
30
20
10
50
100
150
200
250
PROTEIN (µg)

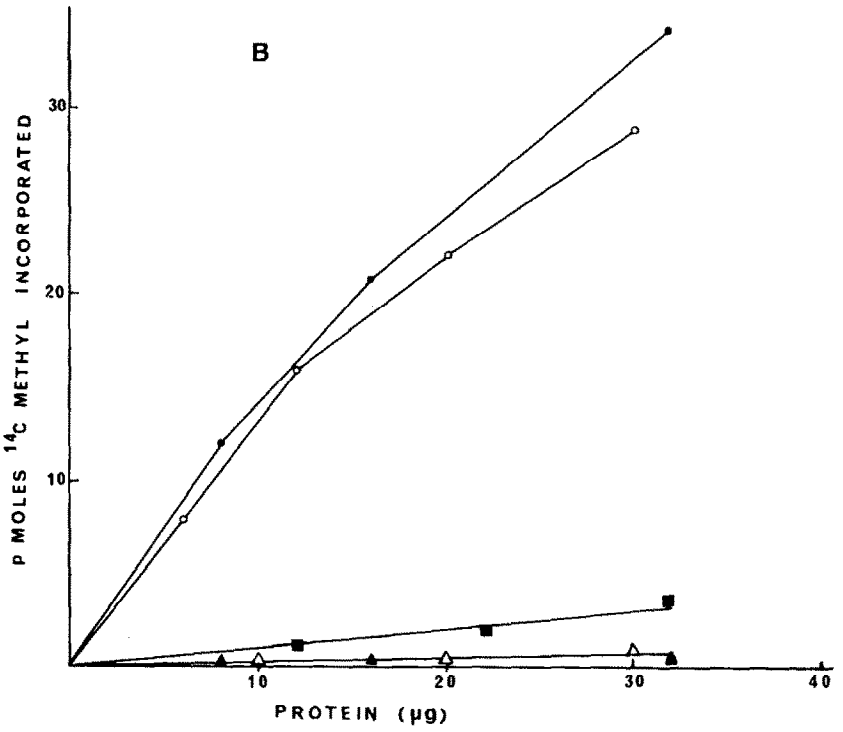

B
p MOLES ^{14}C METHYL INCORPORATED
30
20
10
10
20
30
40
PROTEIN (µg)

Fig.2a, 2b

best substrate among the five different proteins tested. As gp 35 purified by the filtration method is not entirely free of gp 80 [18] it is difficult to speculate about the number of methylation sites in the two glycoproteins individually.

Electrophoretic analysis of the in vitro methylated virus was also tempted. As the methyl ester is stable only under acidic conditions, the method of Gagnon et al. was used [6]. Electrophoresis was performed in the presence of *N*-cetylpyridinum chloride (CPC) as detergent in 3% acetic acid. The four proteins p 27, p 19, p 15 and p 12 migrated in the gel but the radioactive proteins remained at the origin. [^{3}H]Glucosamine-labelled glycoproteins of RSV analyzed under the same conditions behave identically. This phenomenon is probably due to the precipitation of glycoproteins in the presence of CPC.

The physiological role of enzymatic methylation of carboxyl groups is well established in the case of bacterial chemotaxis [5]. In eukaryotic cells, this kind of methylation seems to concern signal transduction and control of cell behaviour [5]. The methylation of enveloped viruses seems to be general since five different viruses could be methylated by PM II, in vitro. The fact that only the two glycoproteins among the viral proteins can be methylated, suggests a role for this modification in the life cycle of the virus. Recent studies have shown that purified glycoproteins from different viruses bind specifically to sites on cell membranes with high affinity [19,20]. A possible role of the methylation of virus glycoproteins can be the regulation of this binding by neutralization of the negatively charged free carboxyl groups.

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Fig.2. Methylation of various substrates by PM II purified from chick embryo. (a) Increasing amounts of proteins were incubated in the standard assay mixture described above, with 15 units of PM II and 2.5 μCi [^{14}C]AdoMet. The extent of methylation was determined as usual. –▲–▲, bovine serum albumin (Sigma); △–△, ribonuclease (Sigma); ■–■, ovalbumin (Sigma); □–□, γ-globulins (Sigma); ●–● RSV; ○–○, purified gp 80. (b) Increasing amounts of purified viral proteins (see Fig.1) were incubated with 15 units of purified PM II and 2.5 μCi [^{14}C]AdoMet and the extent of methylation was determined. Radioactivity due to labelled proteins was deduced from each value. This background represents no more than 10% of total counts in the case of the two glycoproteins. ●–●, gp 80; ○–○, gp 35; ■–■, p 27; ▲–▲, mixture of p 19 and p 15; △–△, p 12 or p 10.