EFFECT OF 5′-METHYLTHIOADENOSINE ON IN VIVO METHYL ESTERIFICATION OF HUMAN ERYTHROCYTE MEMBRANE PROTEINS

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1. Introduction

The enzyme *S*-adenosylmethionine:protein carboxyl-*O*-methyl transferase (EC 2.1.1.24; protein methylase II) methyl esterifies the aspartyl and/or glutamyl residues of preformed proteins, using *S*-adenosylmethionine as the methyl donor [1–4]. The protein carboxyl methyl esters formed are unstable and undergo rapid hydrolysis at physiological pH and temperature yielding methanol [5,6]. Several membrane proteins were shown to be the endogenous substrates for the enzyme in both prokaryotic and eukaryotic cells [1,7–9] and a number of studies were done to determine the physiological roles of this selective, post-translational, protein methylation in membrane functions. The high activity of PM II and the large amounts of its membrane-bound endogenous substrates in nervous and endocrine tissues suggest a role of this reaction in the synaptic function [10] and neurosecretory processes [10–12]. In *Escherichia coli* [8,13] and *S. typhimurium* [9,14] the carboxyl methyl esterification of cytoplasmic membrane proteins appears to be involved in chemotaxis. In chemotactically-active mammalian neutrophils, the methyl esterification of specific membrane-bound chemotactic receptors may initiate the signal to direct the cellular motion for eventual phagocytosis [15]. Thus a relationship may exist between alterations of protein carboxyl methylation and genetic diseases (involving perturbation of AdoMet metabolism) associated with severe immunodeficiency [16].

Abbreviations: MTA, 5′-methylthioadenosine; SIBA, 5′-isobutylthioadenosine; AdoMet, *S*-adenosylmethionine; PM II, *S*-adenosylmethionine:protein-*O*-carboxyl methyl transferase

5′-Methylthioadenosine, a natural nucleoside present in μM levels in mammalian tissues [17], is one of the main products of AdoMet metabolism: in eukaryotic cells, AdoMet is converted into MTA through 3 independent pathways [18]. This thioether exerts an inhibitory effect on a variety of biological systems [18,19]: the inhibitory concentrations of MTA are usually at the same level as it as found in mammalian cells [18,19].

The inhibition exerted in vitro by MTA on PM II is of particular interest [20,21]. To investigate the control mechanism operated by MTA on the enzymatic methyl esterification of membrane proteins in the living cells, the effect of the compound on in vivo methyl esterification of erythrocyte membrane proteins has been analyzed. In these cells, a specific methyl esterification of two membrane components, glycophorin A and 'band 4.5' has been demonstrated in vitro [22,23] and in vivo [23,24]. The inhibition mechanism of MTA in the system investigated has been studied using several analogs and derivatives of the thioether, including 5′-isobutylthioadenosine (SIBA).

A short report of this work appeared in [25].

2. Materials and methods

L-[*methyl*-^{3}H]Methionine (spec. act. 12 Ci/mmol), was from the Radiochemical Centre (Amersham). AdoMet, obtained from cultures of *Saccharomyces cerevisiae*, was isolated by ion-exchange chromatography [26] and MTA was prepared by acid hydrolysis of AdoMet [27]. Adenine, adenosine, ATP, AMP and dibutyryl-cAMP were supplied by Sigma Chemical (St Louis MO). SIBA was supplied from Sefochem, Fine

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Chemicals (Israel). All other chemicals were the purest grade available from standard commercial sources.

2.1. *Methyl esterification of intact erythrocyte membrane proteins*

The carboxyl methyl esterification of erythrocyte membrane proteins was done in vivo as in [24] with minor modifications. The methyl labelling of the cells was done in 0.5 ml total vol. containing 0.1 ml freshly washed erythrocyte, 62.5 μmol sodium phosphate (pH 7.4), 6.4 μmol $MgCl_2$ and 2.5 μmol L-[*methyl*-^{3}H]-methionine (30 μCi). The mixture was incubated at 37°C for 60 min and the extent of methyl esterification of the membrane proteins estimated as in [24].

In the experiments with phosphate-depleted erythrocytes, 1 ml packed red blood cells was washed several times with 300 ml Tris–HCl 5 mM, 0.15 M NaCl (pH 7.4). Cells were labelled as above except that the phosphate buffer was replaced by Tris–HCl buffer.

2.2. *Estimation of L-[*methyl-^{3}*H]methionine and S-adenosyl-L-[*methyl-^{3}*H]methionine in the erythrocytes*

L-[*methyl*-^{3}H]Methionine and methyl-labelled AdoMet, formed by the action of AdoMet synthetase during the incubation, were evaluated by HPLC analysis of the erythrocyte soluble fraction as in [28].

2.3. *Evaluation of the rate of hydrolysis of [*^{3}*H]methyl-esterified membrane proteins*

After the above methyl-labelling the erythrocytes were washed several times with phosphate-buffered saline to remove the unreacted [*methyl*-^{3}H]methionine. The washed cells were then divided into 2 similar aliquots. One was hemolyzed and the membranes, isolated as in [22], were incubated at 37°C for various times, in the absence and in presence of 5 mM MTA. The second aliquot of intact erythrocytes was incubated under the above conditions immediately after washing. The rate of hydrolysis of the methyl-esterified groups was estimated as in [29].

2.4. *Other analytical methods*

MTA phosphorylase activity was determined by measuring 5-[*methyl*-^{14}C]methylthioribose-1-P, released from labelled MTA [30]. Protein concentration was estimated by the Lowry method, using bovine serum albumin as standard.

Table 1
Adenine and its derivatives as inhibitors for in vivo carboxyl methyl esterification of human erythrocyte membrane proteins

Compounds	[^{3}H]Methyl incorp. (pmol/sample)	Relative inhibition (%)
None	2.3 ± 0.1	–
Adenine	0.7 ± 0.05	70
Adenosine	1.8 ± 0.1	20
AMP	1.8 ± 0.1	20
Dibutyryl-cAMP	1.8 ± 0.1	20
ATP	2.3 ± 0.1	–
5'-Methylthioadenosine	0.2 ± 0.01	90
Isobutylthioadenosine	0.7 ± 0.05	70

The assay was performed as in section 2 in presence of 5 mM adenosyl-compound; the results are given ± SD

3. Results

Table 1 reports the rate of methyl esterification of erythrocyte membrane proteins after incubation of the cells with different adenine derivatives. At 5 mM only adenine, MTA and its synthetic analog SIBA significantly inhibit the in vivo methylation process. The effect of increasing amounts of these 3 compounds on the methylation reaction is reported in fig.1. At 100 μM MTA inhibits the reaction rate by 50%. The inhibition exerted by its analog SIBA is lower and parallels that of adenine.

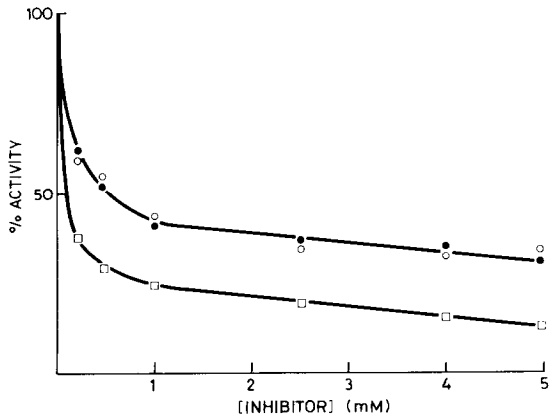

Fig.1. Effect of MTA, adenine and SIBA on in vivo methyl esterification of human erythrocyte membrane proteins. The assay was performed as in section 2 except that the inhibitors were added at the given levels. (○) adenine; (●) SIBA; (□) MTA.

In the erythrocyte cytosolic fraction an active MTA phosphorylase is present which rapidly cleaves MTA and SIBA into adenine and methylthioribose-1-P, and adenine and isobutylthioribose-1-P, respectively [30]. Therefore, to study the inhibitory effect of intact nucleosides on this system, phosphate-depleted erythrocytes have been used. After removal of the endogenous phosphate by repeated washing of erythrocytes with Tris-HCl saline buffer (pH 7.4) the 2 thioethers are not cleaved phosphorolytically [31]. In these phosphate-depleted cells, MTA still has a significant inhibitory effect, whereas SIBA appears to be completely ineffective (fig.2). This result indicates that the inhibition exerted by the synthetic analog should be ascribed to the action of the adenine product of phosphorolytic cleavage of SIBA. These data confirm the lack of any inhibition exerted in vitro by this drug on purified PM II [20]. The lower inhibition exerted by MTA in the phosphate-depleted red blood cells (25% at 100 μM MTA) indicates that in the intact erythrocytes the action of this thioether is probably due to the additive effects of the molecule itself and of the adenine product of its enzymatic cleavage.

To clarify the nature of the inhibition exerted by MTA on this reaction the effect of the thioether on various biochemical processes involved in the in vivo methyl esterification of membrane proteins has been analyzed. In the system investigated it is assumed that the labelled methionine will be transported through the membrane, will be enzymatically activated to yield AdoMet, and that the membrane proteins will be then methylated by the endogenous soluble PM II. MTA does not affect the methionine-transport system in erythrocytes, as estimated from [*methyl*-^{3}H]methionine content of the intact cells after incubation in the presence of labelled methionine and increasing amounts of MTA (not shown). The effect of adenine and MTA on the in vivo synthesis of AdoMet by AdoMet synthetase has been studied by estimating the [*methyl*-^{3}H]-AdoMet content of erythrocytes with HPLC analysis of the soluble fraction after incubation as in section 2. Fig.3 shows that MTA in the standard assay conditions parallels the effect of adenine by increasing the AdoMet synthesis. This effect is probably attributable to the phosphorolytic cleavage of MTA to adenine, which in turn affects cellular ATP levels. In the phosphate-depleted erythrocytes where the MTA phosphorylase is not operative, the thioether does not significantly interfere with the AdoMet synthesis.

The protein methyl esters formed by the action of PM II are known to be unstable at physiological pH and temperature [8]. In fig.4 the rates of hydrolysis of membrane-bound protein methyl esters in intact erythrocytes and isolated erythrocyte ghosts in the absence and presence of 5 mM MTA are compared. The stability of protein methyl esters in intact erythrocytes appears to be slightly higher than that of the

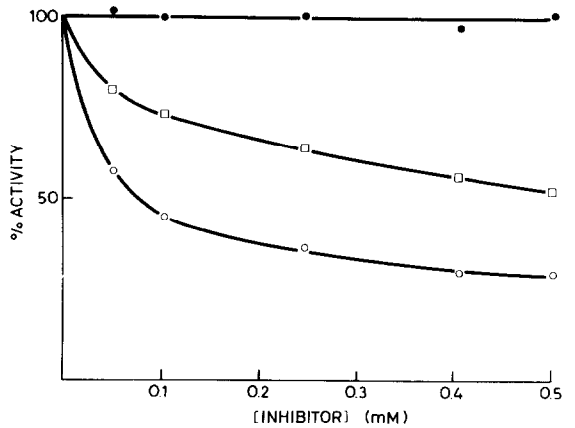

Fig.2. Effect of MTA and SIBA on in vivo methyl esterification of membrane protein in phosphate-depleted human erythrocytes. To remove the endogenous phosphate, the erythrocytes were treated as in section 2. The assay was done as previously described except that the buffer was Tris–HCl 0.15 M (pH 7.4). SIBA and MTA were added at the given concentrations. In the experiments with MTA, the phosphate-depleted cells were also incubated in presence of phosphate buffer 0.15 M (pH 7.4): (•) SIBA; (□) MTA; (○) MTA in phosphate buffer.

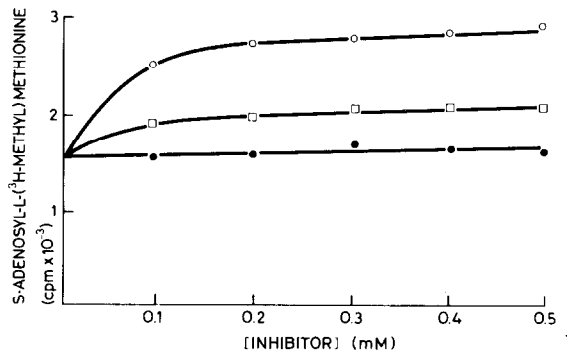

Fig.3. Effect of adenine and MTA on the in vivo synthesis of [*methyl*-^{3}H]AdoMet. The AdoMet levels were estimated by HPLC analysis of erythrocyte soluble fraction as in [28]. The experiments with MTA were done both with normal and phosphate-depleted erythrocytes. For the assay conditions see section 2: (○) adenine; (□) MTA; (•) MTA in phosphate-depleted cells.

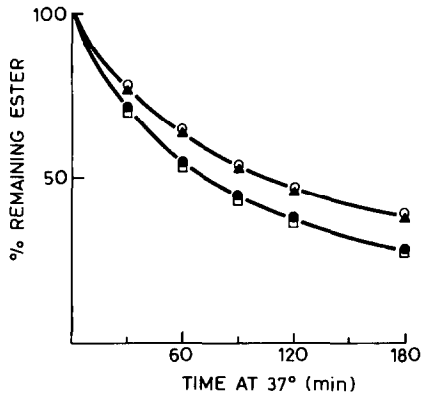

Fig.4. Effect of MTA on the rate of hydrolysis of membrane protein methyl esters in intact erythrocytes and isolated erythrocyte membranes. The assays were carried out as in section 2. In experiments with MTA, the thioether was added at 5 mM: (○) intact erythrocyte protein methyl esters; (▲) intact erythrocyte protein methyl esters in presence of MTA; (□) erythrocyte ghost protein methyl esters; (●) erythrocyte ghost protein methyl esters in presence of MTA.

methyl-esterified isolated membrane, the half-life of methyl esters being 100 min and 70 min, respectively, at pH 7.2. However, in both cases the presence of 5 mM MTA does not modify the rate of hydrolysis, indicating that the thioether is not involved in the de-esterification process.

4. Discussion

This paper reports the in vivo inhibitory effect exerted by MTA on the carboxyl methyl esterification of human erythrocyte membrane proteins. In the system investigated the thioether does not affect the methionine uptake, the enzymatic synthesis of AdoMet nor the rate of hydrolysis of the protein carboxyl methyl esters formed. Therefore, we may infer that in vivo enzyme PM II is directly inhibited by MTA. Since the nucleoside is present at μM levels in mammalian tissues [17,18], and it is actively transported through the biological membranes [31,32] we can postulate a new regulatory role of the molecule on the cellular functions mediated by the enzymatic methyl esterification of membrane proteins (e.g., chemotaxis, neurosecretory processes. In the erythrocyte membrane one of the major methyl acceptor components (band 4.5) has been implicated as a part of the glucose-transport system [33]; therefore, a MTA control mechanism may operate in vivo on sugar transport in the red blood cells.

5′-Isobutylthioadenosine, a synthetic analog of MTA endowed with antiproliferative action [18] which does not inhibit PM II in vitro [20], exerted a significant inhibition on the reaction in the system investigated. The in vivo effect of this drug could be due to the action of the adenine product of phosphorolytic cleavage of SIBA [30].

Despite the broad evidence of antiviral and antiproliferative action exerted by MTA and by its synthetic analogs [18,19,31] the mechanism of action of these molecules is still largely obscure. These effects could be ascribed to the inhibition exerted by these molecules on spermine synthetase [34]. However, the existence of different targets for the cytostatic action of these compounds cannot be ruled out. Inhibitors of macromolecular methylation in vitro generally have antiviral and antimitotic effects in vivo [35]. Of the various post-translational macromolecular methylations, the enzymatic methyl esterification of proteins appears to be of particular biological interest, representing an 'on–off' mechanism for the regulation of several protein activities. The reported significant inhibition of this enzymatic system exerted by MTA and SIBA, together with the finding that SIBA interferes in vivo with several biochemical processes mediated by membrane proteins (e.g., nucleoside and glucose uptake [36]) suggests the hypothesis that protein methylase II could represent one of the target enzymes causing the cytostatic effect of these thioethers.

Acknowledgement

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References

[1] Paik, W. K. and Kim, S. (1980) Protein Methylation, Wiley, New York.
[2] Kim, S. and Paik, W. K. (1970) J. Biol. Chem. 245, 1806–1813.
[3] Diliberto, E. J. jr and Axelrod, J. (1974) Proc. Natl. Acad. Sci. USA 71, 1701–1704.
[4] Kim, S. (1977) in: The Biochemistry of Adenosylmethionine (Salvatore, F. et al. eds) pp. 415–434, Columbia University Press, New York.
[5] Kim, S. and Paik, W. K. (1976) Experientia 32, 982–984.

[6] Gagnon, C. (1979) Biochem. Biophys. Res. Commun. 88, 847–853.
[7] Kim, S., Wasserman, L., Lew, B. and Paik, W. K. (1975) J. Neurochem. 24, 625–629.
[8] Kleene, S. J., Toews, M. L. and Adler, J. (1977) J. Biol. Chem. 252, 3214–3218.
[9] Van der Werf, P. and Koshland, D. E. jr (1977) J. Biol. Chem. 252, 2793–2795.
[10] Eiden, L. E., Borchardt, R. T. and Rutledge, C. O. (1979) in: Transmethylation (Usdin, E. et al. eds) pp. 539–546, Elsevier/North-Holland, Amsterdam, New York.
[11] Gagnon, C., Bardin, C. W., Strittmatter, W. and Axelrod, J. (1979) in: Transmethylation (Usdin, E. et al. eds) pp. 521–528, Elsevier/North-Holland, Amsterdam, New York.
[12] Diliberto, E. J., O'Dea, R. F. and Viveros, O. H. (1979) in: Transmethylation (Usdin, E. et al. eds) pp. 529–537, Elsevier/North-Holland, Amsterdam, New York.
[13] Adler, J. (1979) in: Transmethylation (Usdin, E. et al. eds) pp. 505–509, Elsevier/North-Holland, Amsterdam, New York.
[14] Stock, J. B. and Koshland, D. F. jr (1979) in: Transmethylation (Usdin, E. et al. eds) pp. 511–520, Elsevier/North-Holland, Amsterdam, New York.
[15] O'Dea, R. F., Viveros, O. H., Axelrod, J., Aswanikumar, S., Schiffman, E. and Corcoran, B. A. (1978) Nature 272, 462–464.
[16] Pike, M. C., Kredich, N. M. and Snyderman, R. (1978) Proc. Natl. Acad. Sci. USA 75, 3928–3932.
[17] Seidenfeld, J., Wilson, J. and Williams-Ashman, H. G. (1980) Biochem. Biophys. Res. Commun. 95, 1861–1868.
[18] Zappia, V., Cartenì-Farina, M., Cacciapuoti, G., Oliva, A. and Gambacorta, A. (1980) in: Natural Sulfur Compounds. Novel Biochemical and Structural Aspects (Cavallini, D. et al. eds) pp. 133–148, Plenum, New York.
[19] Ferro, A. J. (1979) in: Transmethylation (Usdin, E. et al. eds) pp. 117–126, Elsevier/North-Holland, Amsterdam, New York.
[20] Oliva, A., Galletti, P., Zappia, V., Paik, W. K. and Kim, S. (1980) Eur. J. Biochem. 104, 595–602.
[21] Oliva, A., Galletti, P., Zappia, V., Paik, W. K. and Kim, S. (1980) in: Natural Sulfur Compounds. Novel Biochemical and Structural Aspects (Cavallini, D. et al. eds) pp. 55–66, Plenum, New York.
[22] Galletti, P., Paik, W. K. and Kim, S. (1979) Eur. J. Biochem. 97, 221–227.
[23] Kim, S. and Galletti, P. (1979) in: Transmethylation (Usdin, E. et al. eds) pp. 547–559, Elsevier/North-Holland, Amsterdam, New York.
[24] Kim, S., Galletti, P. and Paik, W. K. (1980) J. Biol. Chem. 255, 338–341.
[25] Galletti, P., Cartenì-Farina, M., Oliva, A., Manna, C., Della Ragione, F. and Zappia, V. (1980) Abstr. Commun. 13th FEBS Meet., Jerusalem. S1-P50.
[26] Shapiro, S. K. and Ehninger, D. J. (1966) Anal. Biochem. 15, 323–333.
[27] Zappia, V., Zydek-Cwick, C. R. and Schlenk, F. (1969) J. Biol. Chem. 244, 4499–4509.
[28] Zappia, V., Galletti, P., Porcelli, M., Manna, C. and Della Ragione, F. (1980) J. Chromatog. 189, 399–405.
[29] Galletti, P., Paik, W. K. and Kim, S. (1978) Biochemistry 17, 4272–4276.
[30] Cartenì-Farina, M., Della Ragione, F., Ragosta, G., Oliva, A. and Zappia, V. (1979) FEBS Lett. 104, 266–270.
[31] Della Ragione, F., Cartenì-Farina, M., Porcelli, M., Cacciapuoti, G., Gragnaniello, V. and Zappia, V. (1981) Ital. J. Biochem. in press.
[32] Zappia, V., Galletti, P., Porcelli, M., Ruggiero, G. and Andreana, A. (1978) FEBS Lett. 90, 331–335.
[33] Zoccoli, M. A., Baldwin, S. A. and Lienhard, G. E. (1978) J. Biol. Chem. 253, 6923–6930.
[34] Pajula, R. L. and Raina, A. (1979) FEBS Lett. 99, 343–345.
[35] Borchardt, R. T. (1980) J. Med. Chem. 23, 347–357.
[36] Pierré, A. and Robert-Géro, M. (1979) FEBS Lett. 101, 233–238.