Differential membrane protein carboxyl-methylation of intact human erythrocytes by exogenous methyl donors

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The patterns of membrane protein carboxyl-methylation by protein methylase II (*S*-adenosylmethionine:protein-carboxyl *O*-methyltransferase, EC 2.1.1.24) in intact human erythrocytes were shown to differ markedly whether the methyl donor, *S*-adenosyl-L-[methyl-³H]methionine, was supplied exogenously or formed intracellularly via exogenously added L-[methyl-³H]methionine. The differences include the following. (1) The methylation of cytoskeletal components (band 2.1 and 4.1) occurs only in the case of the L-[methyl-³H]methionine-labelled cells. (2) The methionine-mediated methylation was much less sensitive to *S*-adenosyl-L-homocysteine inhibition than the adenosylmethionine-mediated methylation (22% versus 95% inhibition at 10 μ M). (3) The membrane protein methylation mediated by exogenous adenosylmethionine and methionine differed markedly in their alkali labilities; at pH 6.0, 30% of the adenosylmethionine-mediated protein methyl esters were hydrolysed after 30 min (37°C) while the methionine-mediated esters were stable. At pH 7.4, the respective labilities were 60% and 30% for the 30 min incubation. To explain these results, a possible involvement of cytoskeletal structure associated with the intact erythrocyte is discussed.

The enzymic methylation of acidic side chains of glutamyl and aspartyl residues in protein methylase II (*S*-adenosylmethionine:protein carboxyl *O*-methyltransferase, EC 2.1.1.24), has been the subject of much study in recent years (Diliberto *et al.*, 1976; Kleene *et al.*, 1977; Springer & Koshland, 1977; Janson & Clarke, 1980; Kim *et al.*, 1982). The reaction, although not generally well understood in terms of its function, neutralizes negative charges and is thus potentially able to alter protein structure and function. Furthermore, carboxyl-methyl esterification is readily reversible under physiological condition and is an attractive means of regulating a protein's function in a transitory manner. Indeed, the methylation of specific membrane proteins at glutamyl residues has been definitively shown to be integral to the regulation of bacterial chemotactic behaviour (Kleene *et al.*, 1977; Springer & Koshland, 1977).

Erythrocytes are well known to possess high levels of the methylase (Kim, 1974) and endogenous methyl acceptor proteins (Galletti *et al.*,

1979; Terwillinger & Clarke, 1981). Specific membrane proteins have been identified as methyl acceptors by incubation of isolated erythrocyte membranes in the presence of the enzyme and the methyl donor AdoMet. Similarly, incubation of intact erythrocytes with L-methionine (which is activated to AdoMet intracellularly) also showed specific membrane protein methylation to occur.

Recently, it has been observed that the specificity of erythrocyte membrane methylation is not exclusively a function of the intrinsic characteristics of the proteins. The observation of cytoskeletal component methylation in intact cells incubated with L-[methyl-³H]methionine, but not in broken cells incubated with [methyl-³H]AdoMet, suggested that membrane and/or cellular integrity exerts a marked influence in the substrate specificity (Freitag & Clarke, 1981; Terwillinger & Clarke, 1981). In our present study, we have carefully assessed methylation of both intact erythrocyte and isolated erythrocyte membranes utilizing AdoMet [permeant to erythrocytes (Stramentinoli *et al.*, 1978)] or L-methionine as methyl donors. Additionally, we have shown that the methylation patterns of membrane components in intact cells, but not isolated membranes or broken

Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; SDS, sodium dodecyl sulphate; PBS, phosphate-buffered saline (0.15M-NaCl/5mM-sodium phosphate, pH 7.4).

cells, differs markedly when L-methionine or AdoMet is used as a methyl donor. From our results and the known presence of AdoMet in plasma *in vivo* (Salvatore *et al.*, 1971; Stramentinoli *et al.*, 1978), therefore, we propose the existence of a novel specificity determinant for erythrocyte membrane component methylation.

Experimental

Materials

[Methyl-³H]AdoMet (sp. radioactivity 78.5 Ci/mmol), L-[methyl-³H]methionine (sp. radioactivity 15 Ci/mmol), L-[methyl-¹⁴C]methionine (sp. radioactivity 50 mCi/mmol) and L-[2(n)-¹⁴C]methionine (sp. radioactivity 46.6 mCi/mmol) were purchased from Amersham Nuclear. AdoMet, AdoHcy and cycloleucine were obtained from Sigma. All other chemicals were from various local sources and of the best grade available. Protein methylase II was purified from normal human blood by AdoHcy-Sepharose affinity chromatography as described by Kim *et al.* (1978). All the blood samples used for the experiments were from healthy normal individuals.

Methyl-labelling of membrane with [methyl-³H]AdoMet or L-[methyl-³H]methionine in intact erythrocytes

Freshly obtained heparinized blood was centrifuged at 2000g for 10 min to pellet the cells. The packed cells were then washed three times with 10 vol. of cold PBS. Methyl-labelling of the cells was performed in a total volume of 1.0 ml containing 0.5 ml of packed cells and 0.5 ml of PBS containing 0.127 nmol of [methyl-³H]AdoMet (10 μ Ci) or 12.7 nmol of L-[methyl-³H]methionine (190 μ Ci). An aliquot of radioactive compounds was dried under a gentle stream of purified N₂ at 20°C and then used for the methylation. The reaction mixture was incubated at 37°C for the indicated times. The reaction was terminated by addition of 30 vol. of cold 5 mM-sodium phosphate buffer, pH 7.4. The samples were centrifuged at 27000g for 20 min, followed by washing the pellet five times with 5 mM-phosphate buffer, pH 6.8. This preparation usually contained 3–5 mg of protein/ml.

Separation of polypeptides by SDS/polyacrylamide-gel electrophoresis

Methylated membranes (150 μ g of protein) were electrophoresed in a pH 2.4 sodium phosphate buffer system according to the method described by Fairbanks & Avruch (1972). The low pH of this gel system minimized hydrolysis of esters during the course of electrophoresis. Samples were dissolved in 40 mM-dithiothreitol/1% SDS/10 mM-

Tris/HCl (pH 7.0)/1 mM-EDTA/10% sucrose containing 10 μ g of Pyronin/ml as a tracking dye, and incubated at 37°C for 20 min. The electrophoresis was performed on a 3 mm-thick slab gel consisting of 5.6% acrylamide and 0.19% *NN'*-methylenebisacrylamide in an apparatus similar to that described by Studier (1973) at 60 V for 4 h or until the tracking dye migrated to 10 cm from the origin. The gels were routinely stained for proteins with Coomassie Brilliant Blue G and destained according to Fairbanks *et al.* (1971). The radioactivity incorporated into the individual polypeptides was visualized by fluorography of Coomassie Blue-stained and dried gel. The gels were enclosed with pre-exposed Kodak (X-Omat R) for 7–9 weeks at –70°C.

Results

Methylation of intact erythrocyte membranes with exogenous AdoMet and methionine

Intact erythrocytes were methylated with two different radiolabelled methyl donors, AdoMet and methionine. The former has recently been shown to be permeant to the plasma membrane and serves as a methyl donor in the trans-methylation of cellular components (Stramentinoli *et al.*, 1978; Zappia *et al.*, 1978). The latter must be activated intracellularly to form AdoMet before ultimately serving as methyl donor. Since protein methylase II is present intracellularly as a cytosolic enzyme (Kim, 1974), we initially supposed that AdoMet should act similarly either when supplied to the intracellular compartment from the outside or when formed intracellularly from methionine. However, as shown in Fig. 1, the pattern of incorporation of radioactivity into membrane polypeptides differed markedly, as analysed by polyacrylamide-gel electrophoresis and subsequent radioautography. Over the time of incubation with exogenous AdoMet, incorporation was observed into a 48 kDa band at 20 min (lane *a*) and then into a 105 kDa band at 40 min and thereafter (lanes *b–d*). At 150 min (lane *d*), additional incorporation into bands of 76 kDa and 55 kDa was apparent while the incorporation into the 48 kDa band was somewhat diminished in intensity (probably as a result of de-esterification). On the other hand, with methionine, the incorporation profile was quite different; polypeptides at 200 kDa (band 2.1), 81 kDa (band 4.1) and 48 kDa were radiolabelled at 40 min (lane *g*) and 60 min (lane *h*). At 150 min, several additional bands were also shown to be labelled (lane *i*); thus the major radioactive bands were of 200, 105, 81, 55, 48 and 35 kDa. It should be noted, however, that the differences observed were not due to anomalies in the gel separation, since in all lanes the Coomassie Blue staining patterns were essentially identical. It is important to mention

Fig. 1. SDS/polyacrylamide slab gels of erythrocyte membrane proteins methylated *in vivo*. Intact erythrocytes (0.5 ml) were incubated in an equal volume of PBS with [methyl- ^{14}C]AdoMet (10 μCi) or L-[methyl- ^3H]methionine (190 μCi) at 37°C for the indicated time and the membranes were prepared as described in the text. The methyl- ^3H -labelled membranes, containing 150 μg of protein, were electrophoresed on SDS/5.6% polyacrylamide slab gels as described (Studier, 1973). Lanes of radioautography (left) and Coomassie staining (right) are shown. (a)–(e), incubation with [methyl- ^3H]AdoMet; (f)–(j), incubation with [methyl- ^{14}C]methionine; (a) and (f), incubation for 20 min; (b) and (g), for 40 min; (c) and (h), for 60 min; (d) and (i), for 150 min; (e) and (j), for 150 min with 12.5 mM-cycloleucine. The numbers in the left margin indicate standard molecular mass markers and in the right margin refer to the major erythrocyte polypeptide bands according to the nomenclature of Steck (1974).

further that no haemolysis occurred during the incubation, as judged by haemoglobin released (measured by absorbance at 420 nm), nor was protein methylase II activity detected in the extracellular supernatant, indicating that the cell remained intact. Therefore, a possibility of methylation on the outer face of membrane proteins upon haemolysis can be ruled out.

Evidence for methionine-mediated methylation via AdoMet

Although the [methyl- ^{14}C]methionine-mediated incorporation of radioactivity into membranes has been shown to be via transmethylation, this was further confirmed in the present study by the following criteria. (1) When L-[2(*n*)- ^{14}C]methionine was used in place of [methyl- ^3H]methionine, the incorporation into membrane was less than 10% of that observed with the latter (results not shown). (2) Inclusion of cycloleucine, an inhibitor of AdoMet synthetase (Lombardini *et al.*, 1970), in a 2.5 h incubation totally prevented the appearance of radioactivity in all bands (compare lanes i and j of Fig. 1). (3) Addition of increasing amounts of unlabelled AdoMet to the [methyl- ^3H]methionine incubation mixture diminished progressively the amount of radioactivity incorporated into membranes (Fig. 2). Finally, (4) Ado-Hcy, a transmethylation inhibitor, inhibited the methionine-mediated incorporation. These results are shown in Table 1 and Fig. 3, and are described in another context below.

Fig. 2. Effect of unlabelled AdoMet during the methylation of intact erythrocytes with L-[methyl- ^3H]methionine. The washed intact erythrocytes (0.5 ml) were incubated in an equal volume of PBS containing L-[methyl- ^3H]methionine (190 μCi) with the indicated amount of unlabelled AdoMet at 37°C for 150 min. The membranes were prepared as described in the text and counted for radioactivity incorporated.

Further characterization of membrane protein methylation mediated by AdoMet or methionine on intact erythrocytes

In order to understand better the difference between the methyl labelling patterns with AdoMet and methionine, the inhibitory capability of

AdoHcy [a strong competitive inhibitor for methyltransferases (Zappia *et al.*, 1969; Kim, 1974)], on the two types of methylation was examined. As shown in Table 1, methyl-labelling with [*methyl*-³H]AdoMet is much more sensitive to the inhibitor than that with L-[*methyl*-³H]methionine; 95% versus 22% inhibition respectively at 10 μ M-AdoHcy concentration. This relative insensitivity of methionine-mediated methylation was observed even at 200 μ M concentration of the inhibitor.

The inhibition of the methylation of individual polypeptides by AdoHcy was further assessed by

Table 1. *Inhibitory effect of AdoHcy on intact erythrocyte membrane protein methylation with [*methyl*-³H]AdoMet or [*methyl*-³H]methionine as exogenous methyl donor*
Intact erythrocytes were incubated either with [*methyl*-³H]AdoMet or L-[*methyl*-³H]methionine in the presence of the indicated amount of AdoHcy at 37°C for 20 min and the methylated membranes were isolated as described in the text. The amount of [³H]methyl incorporation into the membranes in the absence of AdoHcy with [*methyl*-³H]AdoMet and with [*methyl*-³H]methionine were 104200 c.p.m./mg and 88000 c.p.m./mg, respectively.

AdoHcy (μ M)	Inhibition (%)	
	With [<i>methyl</i> - ³ H]AdoMet	With [<i>methyl</i> - ³ H]methionine
0	0	0
10	95.0	22.0
50	98.3	25.1
200	98.9	33.8

SDS/polyacrylamide-gel analysis, followed by autoradiography of the gels. As shown in Fig. 3, lane *b*, an essentially complete inhibition of AdoMet-mediated methyl incorporation into the various peptides was observed at only 5 μ M-AdoHcy. On the other hand, in the case of methionine-mediated methylation, only a partial inhibition was noted at a concentration of 50 μ M-AdoHcy (lane *e*). Even at 5 mM-AdoHcy, methyl incorporation was not completely inhibited (lane *h*).

The methylation of erythrocyte membrane in the intact cells mediated either by AdoMet or by methionine were also compared for their relative alkali lability. Intact cells were methylated utilizing the two *methyl*-³H donors, and the membranes were prepared. These membranes were subsequently subjected to treatment at various pH values (6.0, 7.4 and 10.0) and the amount of remaining ester was measured during the course of the treatment (Fig. 4). To our surprise, the stability of protein methyl esters in the two preparations was remarkably different. The methyl esters arising from AdoMet were much more labile than those arising from methionine. The difference in lability was greater at pH 6.0 than at pH 7.4 and 10.0. Specifically, at pH 6.0 more than 30% of the AdoMet-derived methyl groups were hydrolysed in 30 min whereas the methionine-derived methyl groups were essentially stable. Furthermore, when membrane methyl esters were hydrolysed for 5 min at pH 7.4 and 100°C, approx. 95% of the AdoMet-derived methyl groups were hydrolysed, while only 80–85% of the methyl groups arising from methionine were labile.

Fig. 3. *SDS/polyacrylamide slab gels with membrane proteins methylated in the presence of AdoHcy*
Intact erythrocytes were incubated with [*methyl*-³H]AdoMet or L-[*methyl*-³H]methionine for 150 min at 37°C in the presence of the indicated amount of AdoHcy and the membranes were isolated as described in the text. The *methyl*-³H-labelled membranes, containing 150 μ g of protein, were electrophoresed on SDS slab gels. Identical lanes of radioautography (left) and Coomassie-stained polypeptides (right) are shown. Symbols at bottom indicate different concentrations of AdoHcy with [*methyl*-³H]AdoMet (*a*–*c*) and with L-[*methyl*-³H]methionine (*d*–*h*); (*a*) and (*d*) contained no AdoHcy; AdoHcy concentrations were 5 μ M (*b*), 50 μ M (*c* and *e*), 200 μ M (*f*), 2.5 mM (*g*) and 5.0 mM (*h*). Lanes (*i*) and (*j*) indicate radioautographs of *methyl*-³H-labelled membranes prepared from broken cells with [*methyl*-³H]AdoMet (*i*) and with L-[*methyl*-³H]methionine (*j*) in the absence of AdoHcy.

Fig. 4. Rate of hydrolysis of methylated membranes from cells incubated with [methyl- ^3H]AdoMet or L-[methyl- ^3H]-methionine

The washed intact erythrocytes (0.5 ml) were incubated in an equal volume of PBS with 0.127 nmol (10 μCi) of L-[methyl- ^3H]AdoMet (—, filled symbols) or 12.7 nmol (190 μCi) of L-[methyl- ^3H]-methionine (---, open symbols) at 37°C for 60 min and then the membranes were suspended in an equal volume of the respective buffers: 0.1 M-citrate buffer for pH 6.0 ($\blacktriangle$, $\triangle$), PBS for pH 7.4 ($\bullet$, $\circ$) and 0.1 M-Tris/HCl for pH 10.0 ($\blacksquare$, $\square$), and were incubated at 37°C for the indicated period. At each time points, aliquots (0.03 ml) were withdrawn from each mixture and quenched with 0.03 ml of 0.2 M-HCl and lyophilized to remove [^3H]methanol formed upon hydrolysis. After lyophilization, the residues were dissolved in 0.5 ml of 50 mM-sodium acetate/0.25% Triton X-100, pH 5.2, and counted for radioactivity remaining. Values of 100% radioactivity remaining are 4620 c.p.m. for ($\blacktriangle$) and 2980 c.p.m. for ($\triangle$) at pH 6.0, 3820 c.p.m. for ($\bullet$) and 2320 c.p.m. for ($\circ$) at pH 7.4, and 3470 c.p.m. for ($\blacksquare$) and 2170 c.p.m. for ($\square$) at pH 10.0.

Discussion

Post-translational methyl modification of proteins is catalyzed by protein methyltransferases, and the immediate methyl donor is AdoMet which is biosynthesized from methionine (Kim & Paik,

1965; Paik & Kim, 1980). Both AdoMet and methionine are used interchangeably for the studies of transmethylation reactions. However, methionine is preferred for use as methyl donor for studies *in vivo*, because the cell is more permeable to it than to AdoMet (Zappia *et al.*, 1978). In a study of an uptake of AdoMet with rabbit erythrocytes, Stramentinoli *et al.* (1978) have identified the internalized compound as unmetabolized AdoMet. In the present study, we have also confirmed an uptake of AdoMet by the erythrocytes. When the intact erythrocytes were incubated with [methyl- ^3H]AdoMet, the specific radioactivity of the intracellular AdoMet gradually increased during the 120 min of incubation at 37°C (J.-Y. Ro & S. Kim, unpublished work). These results, together with the inhibitory effect of AdoHcy (a highly potent competitive inhibitor for AdoMet-dependent reactions) on the AdoMet-mediated membrane protein methylation (Table 1 and Fig. 3), strongly suggest that the observed methylation is due to AdoMet-dependent transmethylation reaction.

Despite the presumption that AdoMet derived from two different routes, one internalized directly from the extracellular compartment, and the other biosynthesized *in situ* from methionine, would be able to methylate membrane components from a common cellular pool, our present studies indicate that, when intact human erythrocytes were treated with AdoMet or methionine, methylation of membrane components occurred with a different selectivity. The major most important difference was shown to be the methylation of cytoskeletal protein components (bands 2.1 and 4.1) (Lux, 1979); with AdoMet as the exogenous methyl donor these were not methylated, while they were the major species to be methylated with methionine (Fig. 1). This lack of methylation of cytoskeletal proteins has also been shown previously in broken cells with AdoMet (Terwillinger & Clarke, 1981) and in the present study either with methionine or AdoMet (Fig. 4, lanes *i* and *j*). Therefore, it appears that the methylation of cytoskeletal proteins occurs only under one set of conditions: when intact cells are methylated with methionine but not with AdoMet as the exogenously added methyl donor. This difference in methyl-donor-dependent labelling patterns was also observed with the membranes prepared with a buffer containing proteinase inhibitors (0.5 mM-phenylmethanesulphonyl fluoride and 1 mM-EDTA) (J.-Y. Ro & S. Kim, unpublished work). This indicates that the observed difference was not caused by the action of proteinases.

Based on these observations, we envision that at least two criteria govern the recognition of cytoskeletal components as methyl acceptors. For one,

as Freitag & Clarke (1981) have previously suggested, and also supported by our present work, these cytoskeletal proteins are recognized as acceptors only in their native state. In the process of breaking the erythrocyte by freeze-thawing or in the isolated membranes, this cytoskeletal network is disrupted in some way that renders bands 2.1 and 4.1 unrecognizable as methyl acceptors. The second criterion is the involvement of the location of the methyl donor relative to the location of the acceptor protein. The involvement of this criterion is somewhat unexpected, since it would be surmised that AdoMet, once having entered the cell, would be indistinguishable from the AdoMet formed intracellularly. The best rationalization of the results appears to be that internalized AdoMet, although able to serve as a methyl donor, encounters a second barrier that prevents free diffusion into the area where cytoskeletal methylation and presumably AdoMet synthesis are occurring. While it is difficult for us to define exactly what this barrier might be, it is tempting to speculate that the cytoskeletal network itself, which covers the entire inner surface of the erythrocyte lipid bilayer [Lux, 1979], could produce a hindrance towards AdoMet molecules trying to penetrate deeper into the intracellular milieu. Several other results in our study provide support for the existence of such a barrier. The methionine-mediated methylation is quite insensitive to AdoHcy inhibition in regard to both gross incorporation (Table 1) and individual methylated proteins as well (Fig. 3). On the contrary, AdoMet-mediated methylation is very sensitive. Since AdoHcy is invariably a powerful inhibitor of protein methylation in solubilized reaction mixtures, one must conclude that a secondary barrier (which acts similarly on entrance of AdoMet) prevents the AdoHcy from entering the area in which methylation of cytoskeletal proteins (and several other proteins) is occurring. Only extremely high concentrations of exogenous unlabelled AdoMet (relative to the intracellular concentration of AdoMet) are able to affect a reduction in the incorporation of methionine-derived radiolabel, again an indication of a barrier.

Aside from the novel nature of our findings, it is also important to emphasize that the involvement of AdoMet localization in the specificity of methylation is physiologically relevant. Although protein methylase II exists exclusively inside the erythrocytes (Kim, 1974; Kim *et al.*, 1975) and not in the plasma, AdoMet is present intracellularly and in the plasma as well (Salvatore *et al.*, 1971; Baldessarini *et al.*, 1979). These two pools of AdoMet, therefore, can serve to methylate different subsets of polypeptides *in vivo*. The concept of AdoMet compartmentation is not without precedent.

Recently, existence of multiple pools of AdoMet distinguished by their relative rate of metabolic usage has been reported in both yeast and isolated hepatocytes (Farooqui *et al.*, 1983). However, the relationship of these AdoMet pools to the results in the present report is not obvious, since the AdoMet pools in these cells were derived from intracellularly synthesized AdoMet.

Another difference coming from methylation systems is the apparent difference between the stability of protein methyl esters derived either from exogenous AdoMet or from methionine. It is conceivable that this difference might be due to the kind of amino acid being methylated; for example, aspartic acid or glutamic acid residues. Although it has been reported that a major site of carboxyl methylation in human erythrocyte membrane proteins is aspartyl residues (D-configuration) (McFadden & Clarke, 1982), this observation was made with only 8% of the total methyl group that was incorporated (Janson & Clarke, 1980). Thus, glutamyl methyl esters may exist, but not be observed due to their extreme lability; they are selectively labilized during the hydrolysis procedures. In fact, glutamyl- γ -methyl ester and aspartyl- β -methyl ester have shown to differ markedly with respect to their lability under alkaline conditions: the t_1 of the former at pH 8 and 37°C is 300 min whereas that of the latter is 500 min (cf. Table 8 of Terwillinger & Clarke, 1981); at pH 9.5, 8 min and 104 min (Janson & Clarke, 1980), respectively. Alternatively, however, the neighbouring amino acid residues or protein conformation at the site of methylation might cause the different stability of the methyl esters and there are strong indications that enzymically formed protein methyl esters are heterogeneous in terms of their ester linkage stability (Kim & Paik, 1976; Kleene *et al.*, 1977; Gagnon *et al.*, 1978; Terwillinger & Clarke, 1981).

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