Methylation of Erythrocyte Membrane Proteins at Extracellular and Intracellular D-Aspartyl Sites in Vitro

SATURATION OF INTRACELLULAR SITES IN VIVO

Clare M. O'Connor‡ and Steven Clarke

From the Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, California 90024

A cytosolic protein carboxyl methyltransferase (S-adenosyl-l-methionine:protein O-methyltransferase, E.C. 2.1.1.24) purified from human erythrocytes catalyzes the methylation of erythrocyte membrane proteins in vitro using S-adenosyl-[methyl-3H]methionine as the methyl group donor. The principal methyl-accepting proteins have been identified by sodium dodecyl sulfate-gel electrophoresis at pH 2.4 and fluorography as the anion transport protein (band 3), ankyrin (band 2.1), and integral membrane proteins with molecular weights of 45,000, 28,000, and 21,000. Many of the methylation sites associated with intrinsic membrane proteins may reside in their extracellular portions, since these same proteins are methylated when intact intact cells are used as substrate. The number of methyl groups transferred in these experiments is approximately 30 pmol/mg of membrane protein, a value which represents less than one methyl group/50 polypeptide chains of any methyl-accepting species.

The number of methylation sites associated with the membranes is increased, but not to stoichiometric levels, by prior demethylation of the membranes. The additional sites are associated primarily with bands 2.1 and 4.1, the principal methyl acceptors in vivo, suggesting that most methylation sites are fully modified in vivo. Extracellular methylation sites are not increased by demethylation of membranes.

The aspartic acid β-methyl ester which can be isolated from carboxypeptidase Y digests of [3H]methylated membranes is in the unusual D-stereoisomer configuration. Similar results have been obtained with [3H]methylated membranes isolated from intact cells (McFadden, P. N., and Clarke, S. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2460–2464). It is proposed that the methyltransferase recognizes D-aspartyl residues in proteins and is involved with the metabolism of damaged proteins in vivo.

The enzymatic esterification of protein-associated aspartyl and glutamyl residues is a general feature of both prokaryotic and eucaryotic cells (1). Two distinct classes of protein carboxyl methyltransferases (S-adenosyl-l-methionine:protein O-methyltransferase, E.C. 2.1.1.24), both using AdoMet as the methyl donor, have been identified (2). The first class, which has been identified in bacteria, catalyzes the stoichiometric methylation of glutamyl residues in membrane-bound chemoreceptors and is involved in the regulation of the chemotactic response (2, 3). The second class, which is more widely distributed in prokaryotic and eucaryotic cells (1), demonstrates a low degree of substrate specificity and catalyzes reactions which are markedly substoichiometric (4–6). The physiological significance of these latter reactions is uncertain.

Human erythrocytes contain both carboxyl methyltransferase activity and methyl acceptor proteins (6–12). Since these enucleate cells are not engaged in de novo protein synthesis or nucleic acid methylation, they provide an excellent system for the study of protein methylation reactions in intact cells. Intact cells incubated with L-[methyl-3H]methionine rapidly transport the label into the cytoplasm where it is converted into AdoMet by a cytosolic AdoMet synthetase (13) and utilized in the endogenous methyltransferase reaction. The macromolecular acceptors for the activated methyl groups are almost entirely protein carboxyl groups.

The modified amino acid formed during the reaction(s) in vivo has been isolated from proteolytic digests of human erythrocyte membrane proteins and identified as the β-methyl ester of aspartic acid (14). Subsequent stereochemical analysis revealed a surprising result in that the isolated esters are entirely in the D-stereoisomer (15). These D-aspartyl residues which serve as a substrate have presumably arisen by spontaneous racemization of aspartyl residues in the polypeptide chain. Based on these results, it was suggested that protein carboxyl methylation reactions are involved in the metabolism of damaged proteins.

Fractionation of human (6) or horse (16) erythrocyte cytosol reveals a single protein carboxyl methyltransferase activity associated with a protein which migrates as a monomer with a molecular weight of 25,000 upon gel filtration. In terms of its subunit structure, substrate specificity, and kinetic properties, the erythrocyte enzyme is similar to the methyltransferases which have been purified from other eucaryotic tissues. Each of the isolated enzymes catalyzes the methylation of a large number of protein substrates in a substoichiometric fashion (4, 5).

Previous results have shown that different erythrocyte

* This research was supported by Grant GM 26060 from the National Institutes of Health and by a grant-in-aid from the American Heart Association with funds contributed in part by the Greater Los Angeles Affiliate. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ This work was done during the tenure of an Advanced Research Fellowship of the American Heart Association, Greater Los Angeles Affiliate.

1 The abbreviations used are: AdoMet, S-adenosyl-L-methionine; PBS, phosphate-buffered saline; AdoHcy, S-adenosyl-L-homocysteine; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; AspβMe, aspartic acid β-methyl ester.

2 C. M. O'Connor and S. Clarke, unpublished data.
membrane proteins are methylated in intact (11) and broken cell (10) systems. In this report, we have characterized the products of the reaction catalyzed by the enzyme in vitro using human erythrocyte membranes as the methyl acceptor and S-adenosylmethionine as the methyl donor. The results obtained in this study clarify the nature of the methyl-accepting site by demonstrating that the substrate for the reaction is achieved by a racemized protein. Two classes of such sites are shown to exist. The first class is located on extracellular portions of membrane proteins and these sites would not be expected to be methylated by the cytosolic enzyme in intact cells. The second class of sites is intracellular and reflect sites at which physiological methylation reactions can occur. We also show that the substoichiometric levels of methylation observed in intact cells are not due to rapid demethylation reactions but rather to the limited number of proteins that contain racemized aspartyl residues. These results do not support a regulatory role for carboxyl methylation in erythrocytes but are consistent with a role for this reaction in the repair or degradation of age-racemized proteins (12, 15).

MATERIALS AND METHODS

Preparation and Incubation of Intact Human Erythrocytes—Heparinized human erythrocytes were obtained from healthy volunteer donors. Plasma and buffy coat were removed after centrifugation of the whole blood. The red cell pellet was resuspended in 10–20 volumes of ice-cold PBS (155 mM NaCl, 5 mM sodium phosphate, pH 7.4) and centrifuged for 5 min at 2200 × g. The procedure was repeated twice to wash the cells, and after each wash, the residual buffy coat was removed by aspiration. In some experiments, the washed cells were resuspended to a 20% hematocrit and incubated with agitation at 37 °C for 18 h prior to membrane isolation. The incubation medium consisted of PBS supplemented with 0.167 M glucose, 50 mM L-methionine and 0.1 mg/ml each of penicillin G and streptomycin sulfate. When the cells were to be used as a source of “demethylated” membranes, the medium also contained 5 mM adenosine and 5 mM sulfate. When the cells were to be used as a source of “demethylated” membranes, the medium also contained 5 mM adenosine and 5 mM sulfate.

Preparation of Erythrocyte Membranes—Membranes were prepared by hypotonic lysis of washed and centrifuged red cell pellets in 3 volumes of ice-cold 5 mM sodium phosphate, 1 mM EDTA, pH 7.6. Membranes were collected by centrifugation at 30,000 × g for 10 min, and the supernatant and granulocyte “button” were removed by aspiration. The procedure was repeated three times to wash the membranes. The final pellet was resuspended to a final concentration of 0.25–2.5 mg of protein/ml as determined by a modified Lowry procedure (17) using bovine serum albumin as a standard. Membranes were used immediately or stored at −20 °C before use. Storage of membranes at −20 °C did not affect the results reported here.

Purification of Erythrocyte Carboxyl Methytransferase—Carboxyl methytransferase was purified from erythrocyte cytosol by affinity chromatography on a resin prepared by covalent attachment of the methyltransferase to w-aminohexyl-Sepharose 4B (Sigma) as described previously (18). Briefly, 150 ml of packed erythrocytes were lysed by freezing and thawing in an equal volume of 5 mM sodium phosphate, 5 mM EDTA, pH 7.6, and NaCl. The lysate was centrifuged for 90 min at 17,000 × g, and the supernatant (following reclarification) was applied to a column (15 × 15 cm) of affinity resin. When the column was saturated with methyltransferase, it was washed extensively with 5 mM sodium phosphate, 5 mM EDTA, pH 6.7. The enzyme was eluted from the affinity resin by 10 mM AdoMet (Boehringer Mannheim) in the same buffer. The purified enzyme typically displayed a specific activity of 4000 pmol of methyl groups transferred per min/mg using 0.5 mM ovomucin as substrate at pH 6.0. The protein concentration of the preparation ranged from 20 to 30 mg/ml.

Incubation of Erythrocyte Membranes with Carboxyl Methytransferase—Reaction volumes contained 15 μl of membranes, 10 μl of enzyme (containing 10 μM nonradioactive AdoMet, 0.5–1.0 μl of S-adenosyl-L-[methyl-3H]methionine (15 Ci/mmol, 66.7 μM, Amersham), 4.5–5.0 μl of H2O, and 5 μl of 200 mM buffer salts. In most cases, assays were performed at pH 7.9 using imidazole as the buffering agent, although other buffers were substituted as indicated. Although the pH of the reaction had a profound effect on the results, there is no evidence for direct effects of buffer salts, erythrocyte small molecules, or divalent cations on the reactions. Reactions were carried out at 37 °C in a shaking water bath.

Quantitation of Protein Methyl Esters—Protein-associated methyl esters formed during methyltransferase incubations were defined as acid-precipitable, base-labile, alcohol/toluene-extractable, volatile radioactivity (2). Membrane proteins were precipitated by the addition of 10 ml of cold 10% trichloroacetic acid to the reaction mixture along with 250 μl of 0.1% bovine serum albumin which acted as carrier. The precipitated proteins were pelleted after 30 min on ice by centrifugation at 1080 × g for 9 min. The pellets were washed twice by resuspension in 1 ml of 10% trichloroacetic acid and recentrifugation at 10,000 × g for 10 min. The final precipitates were dissolved in 0.5 ml of 0.5% HCl and analyzed as described previously (2). The precursor proteins were defined as labeled in vitro by the purified methyltransferase and S-adenosyl-L-[methyl-3H]methionine (72 Ci/mmol, 13.9 μM, Amersham) under the conditions described above. The reaction was stopped by the addition of 9.0 ml of sodium citrate buffer (0.2 M citrate, pH 6.0) and the membranes were pelleted by centrifugation at 30,000 × g for 10 min. The membranes (625 μg of protein) were incubated with yeast carboxypeptidase Y (Sigma, C-3888) overnight at 37 °C and the aspartic acid β-methyl ester faction was separated by ion exchange and gel filtration chromatography as previously described (15). The radioactive peak fractions co-migrating with authentic L- and L-aspartic acid β-methyl ester standards were collected and reacted with a 2:1 mixture of L- and D-leucine-N-carboxyamide (15, 21). The product diastereomers, L-Leu-D-Asp β-methyl ester and L-Leu-L-Asp β-methyl ester, were separated on a Beckman 120C amino acid analyzer using an AA-16 resin (0.9 × 53 cm) which was eluted with sodium citrate buffer (0.2 M Na+, pH 3.25). The elution positions of radioactive products were determined by liquid scintillation counting of aliquots in 10 volumes of ACS-II. The elution positions of nonradioactive markers were determined by manual ninhydrin analysis. In these assays, 0.35 ml of each fraction was mixed with 0.15 ml of 4 M NaOH (Baker Chemical) and 0.07 ml of scintillation fluid (22) in a glass test tube (10 × 75). The samples were boiled for 10 min, cooled, and their absorbance was read at 570 nm.

RESULTS

Characterization of the Methyltransferase Reaction in Vitro—It has been previously reported that eucaryotic protein methyltransferase can be purified by a simple procedure which employs affinity chromatography on a resin consisting of AdoHcy covalently linked to Sepharose (18). We have purified the carboxyl methyltransferase from erythrocyte cytosol by a similar procedure and used the enzyme to methylate membrane proteins in vitro. When membrane ghosts are incubated with the purified enzyme and S-adenosyl-L-[methyl-3H]methionine, radioactivity is incorporated
into acid precipitable material which is more than 95% base-labile. The radioactivity liberated by base treatment behaves like authentic methanol in its volatility and extractability into toluene:isoamyl alcohol:methanol. By these criteria, we conclude that the product(s) of the enzymatic reaction are protein methyl esters.

The initial rate of membrane protein methylation in vitro shows a linear dependence on the concentration of enzyme used in the assay (data not shown), and the formation of radioactive product is inhibited by the presence of excess nonradioactive AdoMet (Table I). In addition, the reaction shows end product inhibition by AdoHcy which is characteristic of AdoMet-dependent methyltransferases. From Table I, we can set an upper limit for the $K_i$ for inhibition by AdoHcy at 0.5 $\mu M$, which compares favorably with the reported $K_i$ values for the enzyme (which range from 0.1 to 1.6 $\mu M$ (6, 23)).

The erythrocyte methyltransferase activity is unaffected by the presence of divalent cations or chelating agents such as EDTA in the incubation medium. We have been unable to detect any small molecule effectors in the erythrocyte cytoplasm as well. The inclusion of an ultrafiltrate of red cell molecules with molecular weights less than 10,000 at an equivalent intracellular concentration in the assay had no effect on the number of methyl groups transferred (data not shown).

**Identification of Membrane Protein Methyl Acceptors** — The membrane proteins which serve as substrates for methylation in vitro were identified by SDS-polyacrylamide gel electrophoresis. Fig. 1a shows the fluorogram of the methylated products obtained when the reaction is carried out at pH values ranging from 5.8 to 8.4. It is clear that several membrane proteins are methylated at each pH. At the lower pH values, the principal methyl acceptors are the anion transport protein (band 3) and species with molecular weights of 45,000 and 28,000. All of these appear to be integral membrane proteins, since they are not extracted from the membrane with 10% acetic acid or 40 mM lithium diiodosalicylate (data not shown), treatments which efficiently extract extrinsic proteins (24). Smaller amounts of radioactivity are also incorporated into band 2.1 (ankyrin) and a 21,000-Da species. We also observe a very small amount of incorporation, rep-

![Fig. 1 (left and center). pH dependence of membrane protein methylation in vitro. Membranes were prepared from cells which had been incubated overnight without (a) or with (b) 5 mM adenosine and 5 mM homocysteine thiolactone to inhibit methyltransferase activity. Membranes were incubated with the purified methyltransferase and S-adenosyl-L-[methyl-$^{3}$H]methionine at various pH values (indicated above the lanes). The incubations were buffered with MES-NaOH (5.5-6.6), imidazole-HCl (7.0), HEPES-NaOH (7.4, 7.8) or Tris-HCl (8.2, 8.6). Incubations were carried out at 37 °C for 1 h. Reactions were terminated by boiling in an equal volume of SDS sample buffer. The methylated proteins were separated by electrophoresis and the radioactivity was detected by fluorography. The Coomassie blue-staining pattern of the membrane proteins is not shown but is similar to that depicted in Fig. 5.](http://www.jbc.org/)

![Fig. 2 (right). Effect of mild base treatment of membranes on methyl acceptor sites. Membranes were incubated in 5 volumes of 0.1 M Tris, pH 8.9, for 0, 1, or 2 h as indicated at 37 °C. The membranes were washed with 10 volumes of sodium citrate (0.2 N in Na*, pH 6.0) and resuspended in 5 mM sodium phosphate, 1 mM EDTA, pH 7.6, to a final concentration of 2.5 mg/ml. Membranes were incubated with the methyltransferase and S-adenosyl-L-[methyl-$^{3}$H]methionine at pH 6.6 for 60 min at 37 °C. Methylated proteins were separated by SDS-gel electrophoresis and the radioactivity was detected by fluorography.](http://www.jbc.org/)
Chromotryptsin treatment of intact cells prior to membrane isolation causes a partial loss of the band 3 methylation site with the concomitant generation of a site with a molecular weight of 59,000. This 59,000 species has been previously identified as the NH2-terminal fragment of band 3 (25, 26). A similar digestion with trypsin has no effect on the band 3 staining or radioactivity, as noted previously for intact cells (11). The 45,000- and 28,000-Da proteins are not affected by either the chromotrypsin or trypsin treatments, indicating that they are not the same as erythrocyte glycophorin, which is a substrate for both of these proteolytic enzymes (26).

When the methyltransferase incubation is carried out at increasingly higher pH values, there is a progressive decrease in the total amount of radioactivity associated with membrane polypeptides. This result may reflect both the increased hydrolysis of protein methyl esters at higher pH values as well as the decreased activity of the enzyme, which has a reported optimum of 5.8 with membranes as substrates (8). The most striking differences between the various pH values are in the observed patterns of methylation. As the pH is increased, band 3 methylation comprises a progressively smaller fraction of the total. In addition, there is an increase in the fraction of radioactivity associated with bands 2.1 and 4.1, the principal methyl acceptors in vivo (11, 12). We also observe the incorporation of radioactivity into a 67,000-Da species and into the spectrin bands when the pH of the reaction mixture is 7.8 or greater.

The qualitative differences in membrane protein methylation at the various pH values used here may be due to pH-dependent changes in the conformation of the methyl-accepting proteins themselves. For example, precipitation of membrane cytoskeletal proteins is reported to occur at pH values near 6.0 (27). Alternatively, incubation of membranes at the more basic pH values could generate new sites by hydrolyzing endogenous membrane protein methyl esters. One approach to resolve this question is to use demethylated membranes as substrates for the methyltransferase under similar conditions. In this experiment, shown in Fig 1b, membranes were prepared from cells which had been incubated with 5 mM adenosine and 5 mM homocysteine thiolactone for 18 h prior to membrane isolation. Treatment of intact cells with these compounds increases the intracellular concentration of AdoHcy sufficiently to inhibit the endogenous methyltransferase, thereby allowing the accumulation of unmodified methylation sites (12). Using these demethylated membranes as substrates for the methyltransferase in vivo, we can now detect methylation of bands 2.1 and 4.1 at all pH values (Fig. 1b). Likewise, spectrin (bands 1 and 2) methylation is apparent at all pHs.

Similar results to those in Fig. 1b are obtained when membranes are demethylated by mild base treatment prior to their incubation with the methyltransferase. In these experiments, membranes were first incubated in 0.1 M Tris-HCl, pH 8.9, at 37 °C for 0, 1, or 2 h. The membranes were then washed in cold 0.2 M sodium citrate, pH 6.0, and finally incubated with the methyltransferase at pH 6.6. It is clear from Fig. 2 that the total number of available methylation sites on membranes increases with time of preincubation at pH 8.9. Comparing individual proteins, the largest increases in methyl-accepting activity are associated with bands 2.1 and 4.1, the major sites in intact cells. Smaller increases in methyl-accepting activity are associated with spectrin and the 67,000-Da species. On the other hand, there is only a very small increase in the number of methylation sites associated with band 3 with time of membrane pretreatment at pH 8.9.

Quantitation of the Methyl-accepting Sites Associated with Membranes—The preceding results suggested that most of the physiological methylation sites were saturated with methyl groups in vivo and that removal of endogenous esters was required for the same proteins to serve as methyl acceptors in vitro. By this reasoning, demethylated membranes would be expected to contain more methylation sites than control membranes. This prediction was confirmed by the experiment of Fig. 3 which compares the kinetics of membrane protein methylation for the two sets of membranes. At all time points, there is more radioactivity associated with demethylated membranes and this difference becomes more pronounced at longer times. After 150 min of incubation, 48 and 66 pmol of radioactive methyl groups have been transferred to control and demethylated membranes, respectively. These values are slightly, but similarly, affected by methyl ester hydrolysis. Following the addition of AdoHcy to a final concentration of 100 μM in the incubation medium, some methyl esters are lost from both control and demethylated membranes with half-times of approximately 190 min (data not shown).

Methyl-accepting Sites in Membranes Become Saturated During Incubation with the Methyltransferase—In the experiment of Fig. 3, it is clear that the velocities of the methylation reactions decrease substantially during the 150-min incubation period. Since less than 1% of any membrane polypeptide is modified during this interval, it was important to determine if the reduction in rate was caused by substrate depletion, end product inhibition, or enzyme denaturation. The possibilities were resolved by adding either additional membranes or an equal volume of buffer to the in vitro incubation mixture after 50 min of incubation at 37 °C. From the data of Fig. 4, it is clear that depletion of membrane substrate sites must be occurring, since the rate of methyl group incorporation increases dramatically when additional membranes are added to the mixture. This increase is similar in magnitude to the rate observed at the beginning of the incubation, suggesting that there have been no significant changes in the velocity due to enzyme denaturation, product accumulation, or AdoMet depletion.
External Labeling of Intact Erythrocytes by the Methyltransferase—It is clear from the foregoing results that the methylation sites associated with membrane proteins could be divided into two distinct classes according to their degree of saturation in intact cells. The first class, represented by spectrin, bands 2.1 and 4.1 and the other in vivo sites, is apparently fully methylated in vivo, and their ability to serve as substrates in vitro depends upon the prior removal of the methyl esters. The second class, which is approximately three times the size of the first class, is not methylated in vivo and its methyl-accepting capacity does not increase upon treatment of the donor cells with adenosine and homocysteine thiolactone. This latter class is represented largely by band 3, 45K, 28K, and 21K, which are methylated in both control and demethylated cells to approximately the same extent. Since all the proteins of this latter class are intrinsic and thus would be expected to have both intra- and extracellular segments, we considered the possibility that the proteins were being methylated on sites exterior to the cell. These sites would not be accessible to the methyltransferase in vivo and would therefore require no prior demethylation to act as substrates in vitro. In the experiment shown in Fig. 5, intact cells were incubated with the methyltransferase and S-adenosyl-L-[methyl-^3H]methionine at physiological ionic strength prior to membrane isolation. Assay of the supernatant for hemoglobin absorbance indicated that the cells were intact at the end of the incubation. From the gel, it is clear that band 3, 45K, 28K, and 21K are methylated on their exterior portions. Smaller amounts of radioactivity are associated with species with molecular weights of 130,000 and 76,000. Furthermore, the specific activity of the methylated proteins was the same in control and demethylated membranes.

Hydrolysis of Protein Methyl Esters at pH 8.9—Previous experiments had indicated that protein methyl esters are hydrolyzed more quickly than free aspartic acid β-methyl ester under mildly basic conditions (10). Similarly, we have characterized the methyl esters formed during the incubation of control and demethylated membranes with the methyltransferase by their rates of hydrolysis at pH 8.9 (Fig. 6). Both hydrolysis curves are kinetically complex, indicating a heterogeneous composition of methyl-accepting sites. From the tangents at the initial and final portions of the curves, it is apparent that the half-times for hydrolysis of the methyl esters formed on control membranes vary widely from less than 5 min to greater than 200 min. The methyl esters formed on demethylated membranes in vitro hydrolyze with half-times ranging from 5 to 900 min. The additional more hydrolytically stable class of esters associated with demethylated membranes must arise from sites which are methylated in intact cells and which become unmodified during the overnight incubation of cells with the methyltransferase inhibitors.

For comparison, we have shown the hydrolysis kinetics for methyl esters associated with membranes which were labeled by incubation of intact cells for 3 h with L-[methyl-^3H]methionine (11). These half-times are also very heterogenous, with most varying from less than 3 min to greater than 260 min. At first glance it seems surprising that the demethylated membranes contain a longer, rather than shorter, lived class of methylation sites than do control membranes. However, it must be considered that the intact cell data are biased toward those sites which turn over rapidly in vivo. When the kinetics of formation and turnover of methyl esters in vivo are measured over longer periods of time, we have determined that the majority of the methyl esters in intact cells turn over with half-times of 1000 min or more. These comparatively stable sites should be the predominant class which becomes available during the incubation of cells with adenosine and homocysteine thiolactone.
Methylated in Vitro—The chemical lability of the rapidly turning over methyl esters formed in vitro by the methyltransferase was characteristic of aspartyl β-methyl esters in polypeptides, but it was necessary to purify the ester product in order to confirm this identity. A radioactive product which co-migrated with authentic D- and L-AspβMe standards was purified from carboxypeptidase Y-digested membranes by ion exchange and gel filtration chromatography as previously described (15). This product was hydrolyzed at pH 10.5 with a half-time of 90 min, a rate that is nearly identical with the authentic AspβMe standards (10). Reaction of the radioactive AspβMe with L-leucine-N-carboxyanhydrase produces a new radioactive peak with migrates on an amino acid analyzer column with authentic L-Leu-D-AspβMe, as shown in Fig. 7. Careful examination of the data reveals that the radioactive peak migrates approximately 3 min faster than the nonradioactive L-Leu-D-AspβMe. This slight isotope effect has been noted previously for both AspβMe and L-Leu-D-AspβMe which contain three tritium atoms in the methyl ester (14, 15). The nature of the radioactive peak co-migrating with L-Leu-D-AspβMe was further verified. First, both the radioactivity and the authentic L-Leu-D-AspβMe co-migrate when the pooled material was fractionated by Sephadex G-15 chromatography. In addition, the radioactivity is base-labile; following incubation of the dipeptide fraction at pH 11.8 for 90 min, all of the radioactivity is recovered upon rechromatography on the amino acid analyzer resin as [3H]methanol. From these results, we conclude that the purified methyltransferase catalyzes the formation of D-AspβMe residues in erythrocyte membrane proteins.

At this point, we cannot rigorously exclude the possibility that a small amount of L-AspβMe, representing less than 10% of the amount of D-AspβMe, is formed during the reaction with the methyltransferase. There is a small peak of radioactivity which elutes 4.5 min before the ninhydrin peak of L-Leu-L-AspβMe. The origin of this material is not clear.

**DISCUSSION**

Previous studies have shown that erythrocyte membrane proteins are methylated by an endogenous AdoMet-dependent carboxyl methyltransferase when intact erythrocytes are incubated in the presence of L-[^3H]methionine (9, 11, 12). Using a pH 2.4 gel system, the principal methyl-accepting membrane proteins in intact cells were identified as the cytoskeletal proteins, bands 2.1 and 4.1 (28), while band 3 and other intrinsic proteins were identified as minor acceptors (11, 12). Kinetic analysis of the labeling of these individual proteins revealed, however, that the methylation of each polypeptide was very substoichiometric (less than 0.02 esters/...
Carboxyl Methylation of Erythrocyte Membrane Proteins

Interestingly, it has been shown that the major physiologically methyl acceptors in the erythrocyte membrane do not become methylated in a lysed cell system consisting of cracked erythrocytes and S-adenosyl[methyl-\(^{15}\)\(^{3}\)H]methionine; the radiolabile methyl esters formed in this system were restricted instead to integral membrane proteins which were identified as band 3 and glycophorin using a pH 4.2 gel system (10). A similar assignment of methyl-accepting activity to intrinsic proteins was made using a purified system containing membranes and the purified methyltransferase, but the identification of individual methyl acceptors was complicated by possible base hydrolysis during electrophoresis (8). The pronounced differences in the membrane proteins which were identified as methyl-accepting substrates in intact cells and in the \(\textit{in vitro}\) systems raised the possibility that the purified carboxyl methyltransferase did not catalyze the methylation reactions observed \(\textit{in vivo}\). In the present work, we have studied membrane protein methylation by the purified methyltransferase \(\textit{in vitro}\) with particular attention to the specificity and stoichiometry of the reactions. The results presented here strongly suggest that the purified enzyme catalyzes protein methylation \(\textit{in vivo}\). We clarify some of the discrepancies between the earlier studies by showing that there are two classes of methylation sites associated with membranes which can be distinguished by their degree of saturation \(\textit{in vivo}\) and most likely by their localization across the membrane bilayer as well. The results indicate that the substoichiometry of the reactions in intact cells is due to a very limited number of substrate sites associated with each membrane methyl-accepting polypeptide. We show that the methyltransferase catalyzes the formation of \(\Delta\)-aspartyl \(\beta\)-methyl esters in proteins and suggest that this unusual stereochemistry explains the infrequent occurrence of protein methylation sites both \(\textit{in vivo}\) and \(\textit{in vitro}\).

Identification of Membrane Methyl-accepting Substrates—When membranes are used as substrate for the methyltransferase \(\textit{in vitro}\), the major methyl acceptors are integral membrane proteins, principally the anion transport protein (band 3) and species with molecular weights of 45,000 and 28,000 (Fig. 1). The identity of the latter proteins has not been established, although we know that they are not glycophorin, since their electrophoretic mobility is unaffected by chymotryptic or tryptic treatment of intact cells prior to membrane isolation. In this regard, our results contradict those of an earlier study which reported that the glycophorin monomer and dimer were the major membrane methyl acceptors for the purified enzyme (8). We do detect smaller amounts of radioactivity associated with a 76,000-Da protein which migrates in our gel system with the major periodic acid-Schiff staining band, the glycophorin dimer. Other minor methyl-acceptors include band 2.1 and intrinsic proteins which migrate on SDS gels with \(M_r\) values of 130,000, 21,000, and 11,000. Interestingly, all of the intrinsic proteins can be methylated on their extracellular portions, since they are methylated when intact cells are used as the substrate for the methyltransferase \(\textit{in vitro}\) (Fig. 5). In fact, we cannot exclude the possibility that all of the methylation sites available to the methyltransferase on fresh erythrocyte membranes, with the exception of band 2.1, are on the external portions of integral membrane proteins.

The pattern of protein methylation that we observe using the purified methyltransferase and unmanipulated membranes as substrate (Fig. 1a) is markedly different from the pattern of protein methylation observed in intact cells labeled with \(\text{L}-[\text{methyl-}\^{15}\text{H}]\text{methionine}\). The inability of the major methyl-accepting proteins \(\textit{in vivo}\) to serve as methyltransferase substrates \(\textit{in vitro}\) may be best explained by their saturation with methyl groups \(\textit{in vivo}\). Removal of endogenous methyl esters either by incubation of intact cells with methyltransferase inhibitors prior to membrane isolation or by mild base treatment of isolated membranes liberates new methylation sites which are associated principally with the band 2.1 and 4.1 polypeptides, the principal methyl acceptors \(\textit{in vivo}\). Only a small increase in the methyl-accepting activity of band 3, a minor acceptor \(\textit{in vivo}\), is observed after demethylation of the membrane substrate (Figs. 1b and 2). Methylation sites on the spectrin polypeptides and a 67,000-Da polypeptide are also exposed by demethylation of the substrate membranes. This is, in fact, the first report of methylation sites associated with spectrin. These are probably physiologically significant, however, since we have been able to detect spectrin methylation in intact cells when the methylated proteins are separated by two-dimensional isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The basis for the substoichiometric nature of the carboxylmethylation reactions \(\textit{in vitro}\) may reside in the unusual \(\Delta\)-aspartyl configuration of the substrate site, which from the results reported here is most likely a \(\Delta\)-aspartyl residue. As shown in Fig. 7, the aspartic acid \(\beta\)-methyl ester that is isolated from the membrane is in the unnatural \(\beta\)-stereocconfiguration. Likewise, the methyl ester isolated from intact cells is in the
From the purification data, we estimate that the methyltransferase repair of the damaged proteins (12, 15).

Variety of substrates can be calculated for other eucaryotic damaged proteins. Some possible functions would include the turnover number is not clear at the present time, one intriguing possibility is that the enzyme catalyzes a second unidentified reaction which is involved with the metabolism of damaged proteins. Some possible functions would include the targeting of damaged proteins for proteolytic destruction or direct repair of the damaged proteins (12, 15). From the purification data, we estimate that the methyltransferase concentration in erythrocyte cytosol is approximately 5 μM, or roughly 20-fold higher than that which we have used in the in vitro experiments described here. This high concentration of intracellular methyltransferase may, in fact, compensate for the apparent slowness of the enzymatic reaction in the intact cell. By thus ensuring that all or nearly all of the intracellular D-aspartyl residues are methylated, the methyltransferase may provide protection against the deleterious structural consequences of protein racemization. From the widespread distribution of similar carboxymethyltransferases in eucaryotic tissues, we would expect that proteins in other cell types are also subject to racemization damage and have therefore evolved with a similar protective capacity.

REFERENCES


The specific activities of the methyltransferase in unfractionated cytosol and the purified fractions are 1.62 and 100 pmol transferred per min/mg, respectively. The protein concentration of cytosol is 320 mg/ml (33).

5 P. N. McPadden, J. Horwitz, and S. Clarke, manuscript in preparation.
Methylation of erythrocyte membrane proteins at extracellular and intracellular D-aspartyl sites in vitro. Saturation of intracellular sites in vivo.

C M O'Connor and S Clarke


Access the most updated version of this article at [http://www.jbc.org/content/258/13/8485](http://www.jbc.org/content/258/13/8485)

Alerts:

- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/258/13/8485.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/258/13/8485.full.html#ref-list-1](http://www.jbc.org/content/258/13/8485.full.html#ref-list-1)