Methylation of Membrane Proteins in Human Erythrocytes

IDENTIFICATION AND CHARACTERIZATION OF POLYPEPTIDES METHYLATED IN LYSED CELLS*

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An in vitro system was developed for studying protein methylation reactions in human red blood cells. Packed erythrocytes were lysed by freeze-thawing in the presence of S-adenosyl[methyl-3H]methionine. Specific incorporation of base-labile methyl groups into the band 3 anion transport protein and the major sialoglycoprotein (glycophorin, periodic acid-Schiff reagent-1) was demonstrated by dodecyl sulfate gel electrophoresis at pH 2.4, selective extractions with Triton X-100 and lithium diiodosalicylate, and protease sensitivity. Two other unidentified intrinsic membrane proteins with $M_r \approx 96,000$ and 23,500 were also methylated. Little radioactivity was incorporated into membrane proteins when membranes were incubated with S-adenosyl-[methyl-3H]methionine in the absence of cytosol. No evidence was obtained for incorporation of methyl label into extrinsic proteins including bands 1, 2, 2.1, 4, 5, 6, or in zone 4.5.

Proteolytic digestion of intact cells and isolated membranes revealed that one site of methylation on the band 3 polypeptide may be at the inner surface of the membrane near the junction of the cytoplasmic domain and the membrane domain.

The rates of hydrolysis of the incorporated methyl groups were characterized over a range of pH values. These rates were compared to those of methyl esterified amino acids and peptides, including aspartic acid β-methyl ester which has been isolated from proteolytic digests of methylated erythrocyte membranes (Janson, C. A., and Clarke, S. (1980) J. Biol. Chem. 255, 11640-11643). We find that the rates of base-catalyzed hydrolysis of β-methyl esters of aspartic acid and γ-methyl esters of glutamic acid are highly sensitive to the presence of substituents on the α-carboxyl and α-amino groups. The rates of hydrolysis of the membrane-incorporated methyl groups are consistent with those of internal aspartic acid and glutamic acid methyl ester residues.

The reversible methyl esterification of glutamyl residues on a group of membrane proteins is an essential feature of the mechanism of chemotaxis of enteric bacteria (1, 2). It appears that similar protein methylation reactions also occur in many mammalian tissues. In these eucaryotic cells, the existence of reversible protein methylation systems has generally been surmised from the presence of enzymes which catalyze the transfer of methyl groups from S-adenosylmethionine to exogenous substrate proteins in linkages which may include esters at the COOH-terminal, at aspartyl, or at glutamyl residues (for a recent review, see Ref. 3). Although some functions have been proposed for mammalian carboxyl methylatation reactions, their overall physiological role is still poorly understood (3).

Kim (4) has shown that the cytosol of human erythrocytes contains an enzymatic activity which catalyzes the incorporation of methyl groups from S-adenosylmethionine into base-labile linkages on ovalbumin and other proteins. Using methyltransferases purified from calf brain or rat red blood cells, and using rat erythrocyte membranes as substrates, Galletti et al. (5) reported that methyl groups co-migrated with bands 3, 4, and 4.5 when membrane polypeptides were fractionated by dodecyl sulfate gel electrophoresis. However, using the same methyltransferases and human erythrocyte membranes as substrates, they found that methyl groups co-migrated only with band 4.5 and the major sialoglycoprotein (6). Preliminary studies have also indicated that radioactive methyl groups co-migrate with band 4.5 and the major sialoglycoprotein after incubation of intact erythrocytes with [methyl-3H]methionine (7).

In order to more precisely identify and characterize potential human red blood cell membrane methyl acceptor species, we have developed an in vitro system where S-adenosylmethionine-dependent methylation reactions can be performed in lysed packed red blood cells under conditions where the concentrations of enzymes, protein substrates, and small molecule effectors can be close to those of in vivo conditions. In these studies, we have utilized a pH 2.4 electrophoretic system which minimizes hydrolysis and has used several independent criteria to identify methylated species. We report here the methylation of band 3 in human erythrocytes and confirm the previous suggestion (6, 7) that the major sialoglycoprotein is also methylated in these cells.

Additionally, in this work we have characterized the rates of hydrolysis of the incorporated methyl groups over a range of pH values and have compared them to the rates of hydrolysis of methyl esterified peptides and amino acids. We report here the first evidence that the hydrolysis of methylated membrane proteins proceeds at rates consistent with those of esterified internal aspartyl and glutamyl residues. These results support the recent demonstration of [methyl-3H]glutamic acid β-methyl ester in proteolytic digests of methylated red blood cell membranes (8).

EXPERIMENTAL PROCEDURES

Preparation of Methylated Membrane Proteins—Red cells were obtained from recently outdated blood (UCLA Blood Bank) and were

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Protein Methylation in Erythrocyte Membranes

washed three times in 150 mM NaCl, 5 mM sodium phosphate, pH 8.0. Unsealed ghost membranes were prepared from washed cells by hypotonic lysis in 5 mM sodium phosphate, pH 8.0, as described by Steck and Kant (9).

Except as otherwise noted, methylation reactions were performed by incubating lysed packed red cells with S-adenosyl-L-[methyl-3H]methionine (300 Ci/mmol in H2SO4, at pH 2.5 to 3.5, Amersham). Cells were lysed by freezing at -70°C in dry ice/isopropanol, and then were incubated on a 37°C shaking water bath for 35 min. In this way, cell constituents were maintained at near-physiological levels and the radioactive substrate was accessible to the cell interior.

S-adenosyl-L-[methyl-3H]methionine was prepared from commercially available L-methionine according to published methods (21). S-adenosyl-L-[methyl-3H]methionine (35 Ci/mmol in H2SO4, at pH 2.5 to 3.5, Amersham). Cells were lysed by freezing at -70°C in dry ice/isopropanol, and then were incubated on a 37°C shaking water bath for 35 min. In this way, cell constituents were maintained at near-physiological levels and the radioactive substrate was accessible to the cell interior.

[3H]Methylated erythrocyte membranes were prepared from the incubation mixture by an initial dilution with 30 volumes of 5 mM sodium phosphate, pH 8.0, for 1 min at 2°C to insure hemoglobin release from the membrane (18). This solution was then diluted with 4 volumes (compared to the original incubation) of 46 mM sodium citrate, pH 5.4, to reduce the pH to 6.8 and minimize hydrolysis of methyl ester linkages. Membranes were collected by centrifugation at 10,000 × g at 2°C in a Sorvall SS-34 rotor. The supernatant and the hard "button" underneath the cells were removed and the entire washing procedure was repeated four additional times. The final pellet, at a protein concentration of ~3.3 mg/ml in 4.3 mM NaPO4, 5.3 mM Na citrate, pH 6.8 was frozen at -70°C. 

Approximately 0.5 ml of membranes were obtained from 1 ml of packed cells.

Markers of Polypeptides by Dodecyl Sulfate Gel Electrophoresis—Electrophoresis in polyacrylamide gels was performed in the 50 mM sodium phosphate, pH 2.4, 1% sodium dodecyl sulfate buffer system described by Fairbanks and Avruch (11). The low pH of this gel system minimized ester hydrolysis during electrophoresis. Samples were prepared in 25 mM sodium phosphate, pH 4.2, 2% sodium dodecyl sulfate, 30 mM b-mercaptoethanol, 8% glycerol, and 0.03% pyronin Y and heated 3 min at 100°C. For the gels shown in Figs. 1-3, samples were electrophoresed overnight (60 V, 90 mA) on 3-mm thick slab gels containing 10% acrylamide and 0.34% N,N'-methylene bisacrylamide in an apparatus similar to that described by Studier (12) until the pyronin Y tracker dye had migrated 12-14 cm. For the gel shown in Fig. 4, samples were electrophoresed for 3 h (75 V, 90 mA) on 1.5-mm thick slab gels containing 7.5% acrylamide and 0.26% N,N'-methylene bisacrylamide until the tracker dye had migrated 12 cm. Marker proteins (Bio-Rad Laboratories) were electrophoresed in parallel lanes and included myosin (Mr = 200,000), b-galactosidase (Mr = 117,000), phosphorylase (Mr = 94,000), serum albumin (Mr = 68,000), ovalbumin (Mr = 43,000), carbonic anhydrase (Mr = 29,000), soybean trypsin inhibitor (Mr = 21,500), and lycosyne (Mr = 14,300). Coomassie blue and periodic acid-Schiff reagent staining reactions were performed according to Fairbanks and Avruch (11). In the case, the reaction was quenched by a 10-fold dilution of the incubation mixture in water at 0°C. Control experiments showed that the rate of hydrolysis in 0.1-1.0 mM NaCl at 0°C was less than 0.2%/h.

Detection of Radiolabeled Polypeptides—Two methods were used to measure the incorporation of radioactivity into polypeptides separated by gel electrophoresis. Fluorography of Coomassie-stained gels using sodium salicylate was carried out as described by Chamberlain (14). Alternatively, wet stained gels were sliced into 1- or 2-mm sections for direct counting. Total radioactivity was measured by incubating gel slices at 30°C for 18 h in 6 ml of a solution of 10% Protosol (New England Nuclear) in Econofluor (New England Nuclear). Counts were corrected for quenching using an internal standard method. Base-labile radioactivity is gel slices was determined in parallel experiments by alkaline hydrolysis in 0.15 M of 2 NaOH at 25°C for 2 h. After hydrolysis, proteins were precipitated with 1 M of 20% trichloroacetic acid and an aliquot of the supernatant (after centrifugation at 9000 × g for 4 min) was counted for radioactivity in 10 ml of Handifluor (Mallinckrodt). This figure, corrected for quenching and the volume counted, was taken to be the base-labile radioactivity. Base-stable radioactivity was determined by counting the pellet fraction in 15 ml of 1% Protosol in Econofluor after an 18-h incubation at 30°C.

Protein Determinations—Protein concentrations were determined by a modification of the Lowry method using bovine serum albumin as a standard (19). Samples containing lithium diodiosalicylate (which precipitates protein, interfering with the assay) were precipitated twice with ice-cold acetone before analysis. Samples containing

Triton X-100 were centrifuged at 5000 × g for 3 min immediately prior to spectrophotometry.

Fractionation of Membrane Proteins with Triton X-100 and Lithium Diodiosalicylate—Membranes were extracted with these compounds essentially as described by Yu et al. (16) and Steck and Yu (17). To one volume of packed methylated membranes (at 3-4 mg of protein/ml) was added 10 volumes of 2% Triton X-100, 5 mM sodium phosphate, pH 6.8, and either 0.2% (v/v) Triton X-100 in 40 mM lithium diodiosalicylate. Both of these compounds were added from Sigma. The solutions were incubated at 0°C for 1 h with either 5 g/ml of trypsin or 250 g/ml of chymotrypsin. Reactions were terminated as above by the addition of phenylmethylsulfonyl fluoride to a final concentration of 2 mM. The cells were then washed three times with 50 mM NaCl, 5 mM sodium phosphate, pH 6.8, and used immediately. Control cells were taken through the entire procedure without protease addition.

Determination of Hydrolysis Rate of S-adenosyl Methionine—A 10-μl aliquot of [3H]methylated membranes (3-4 mg of protein/ml) was diluted to 1 ml of 50 mM NaPO4, pH 7.4, 0.9% NaCl, 5 mM sodium phosphate, pH 8.0, were incubated at 37°C for 3 h with either 5 g/ml of trypsin or 250 g/ml of chymotrypsin. Reactions were terminated as above by the addition of phenylmethylsulfonyl fluoride to a final concentration of 2 mM. The cells were then washed three times with 50 mM NaCl, 5 mM sodium phosphate, pH 6.8, and used immediately. Control cells were taken through the entire procedure without protease addition.

Determination of Hydrolysis Rate of Model Compounds—Hydrolysis rates in strong acid were measured by incubating esters (at concentrations of 0.1-0.5 M) in 6 M HCl at 37°C. Aliquots (0.1 ml) were removed at various times and reacted for 15 min at 25°C with 3.5 ml of 0.6 M H2O2-HCl in 1 ml NaOH to form hydroxy acids from the unhydrolyzed esters (20). The concentration of hydroxy acids was determined after the addition of 4.0 ml of 10% FeCl3-H2O in 0.7 M HCl. The absorbance at 540 nm was read against a water blank. Half-times of hydrolysis were determined from a plot of the logarithm of ester concentration versus time.

Hydrolysis rates in the neutral and alkaline range were determined by the rate of protein release measured in a pH Stat apparatus consisting of a Radiometer Titrigraph, type SBR2c, and a Titrator, type TTT1c. Samples were dissolved in 4.0 ml of distilled water in a reaction vessel maintained at 37°C with a circulating water bath, and a stream of purified nitrogen (50 cm3/min) was passed over the reaction to prevent uptake of atmospheric carbon dioxide. The amount of H+ produced by ester hydrolysis was calculated from the amount of 0.1 M NaOH added to the solution necessary to maintain the pH. Under conditions where the pH was tested greater than the pH at which the hydrolysis did not affect the pH of the above functional groups, the rate of protein production was recorded in the absence of NaOH. Reactions were generally carried out until no additional NaOH was added for several half times, and the calculated from a semilogarithmic plot of NaOH addition versus time.

At pH values where hydrolysis was slow, the t1/2 was determined...
from a plot of the initial rates of hydrolysis versus initial ester concentration. The results were corrected for the nonstoichiometric proton release which occurred in compounds such as leucine methyl ester where the pK_a of the amino group was raised upon hydrolysis. Methyl propionate was obtained from Aldrich, L-glutamic acid γ-methyl ester, L-leucine methyl ester-HCl, and L-proline methyl ester-HCl were obtained from Sigma, and L-aspartic acid β-methyl ester-HCl was obtained from Vega. N-Benzoyl-L-glutamic acid γ-methyl ester and N-benzoyl-L-aspartic acid β-methyl ester were synthesized by a procedure similar to that of Vogel (21). The amino acid ester (1.5 mmol) was dissolved in 5 ml of 10% sodium bicarbonate and 3 mM of benzyl chloride (Aldrich) were added. The mixture was incubated for 1 h at 25°C with stirring. Excess benzoic acid was removed by acidification to pH 2 with HCl. The benzoyl derivatives were crystallized from the aqueous phase at 4°C. Titration of each of these compounds revealed only one equivalent group with a pK_a of ~3.4; infrared spectra were consistent with that of the benzoylated amino acid methyl ester. N-Acetyl-L-leucine methyl ester was prepared by reacting 1.2 mmol of L-leucine methyl ester-HCl with 5.3 mM of acetic anhydride (Mallinkrodt) in 5 ml of 10% sodium bicarbonate for 30 min at 25°C. The products were lyophilized, extracted with chloroform, and the insoluble material (containing NaCl and acetic acid) was used directly in titration studies. The peptides N-benzoyl-L-glutamyl γ-methyl ester glycylamide and N-benzoyl-L-aspartyl β-methyl ester glycylamide were synthesized from the benzoyl esters prepared above and glycyamide using a water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl (Sigma), as described by Hoare and Roshband (22). In a typical synthesis, 100 μmol of the benzoyl ester, 3000 μmol of glycyamide-HCl (Sigma), and 200 μmol of the carbodiimide were dissolved in 4 ml of distilled water and the pH maintained at or below 4.75 with a pH Stat apparatus for 2 h at room temperature. The aspartyl peptide was purified from the reaction mixture (dried by flash evaporation at 60°C) by extraction into 5 ml of 2:1 (v/v) chloroform/methanol. After removal of solvent by flash evaporation, the peptide was crystallized twice from a minimal volume of water at 4°C. The glutamyl peptide was extracted from the flash evaporated reaction mixture into 5 ml of chloroform. After removal of solvent as above, the peptide was dissolved in water and passed over an ion retardation resin (AG11A8, Bio-Rad Laboratories) to remove charged species. The purity of the products was confirmed by the absence of titratable groups, by stoichiometric recovery of amino acids after hydrolysis in 6 M HCl for 18 h at 108°C (determined on a Beckman model 120C amino acid analyzer), and by stoichiometric production of protons upon hydrolysis in mild base.

RESULTS

Protein Methylation in Lyzed Human Erythrocytes

Fig. 1 shows the distribution of radioactivity in the polypeptides of red cell membranes prepared from packed lyzed cells incubated with S-adenosyl-L-[methyl-3H]methionine as described under "Experimental Procedures." Polypeptides with apparent M_r = 96,000, 44,000, and 27,000 incorporated substantial quantities of [3H]methionyl groups. The peak of radioactivity at 96,000 daltons co-migrated with the Coomassie blue-staining band 3 region. No Coomassie blue-staining bands co-migrated with the peak of radioactivity at 44,000 daltons. This radioactive peak did coincide, however, with the major PAS staining polypeptide. The peak of radioactivity at ~27,000 daltons was not associated with any of the major Coomassie blue- or PAS-staining polypeptides. As Table I shows, more than 95% of the total radioactivity incorporated into the red cell membrane is labile under mildly basic conditions (pH 9, 22°C, 4.5 h). The cytosolic fraction, reported to contain a protein methyltransferase (4), was required for alkali-labile protein methylation; when membranes were incubated directly with S-adenosyl-L-[3H-methyl]methionine the incorporation of radioactivity into alkali-labile linkages was greatly diminished while base-stable radioactivity persisted. The latter radioactivity might be accounted for by phospholipid methylation (23). Assuming 7.4 × 10^{-10} mg of membrane protein/red cell (10), ~38,000 methyl groups can be incorporated into base-labile linkages/red cell membrane. This is a minimum estimate of the number of base-labile methyl groups which can be incorporated because there is some isotopic dilution of the S-adenosyl-L-[3H-methyl]methionine by endogenous S-adenosylmethionine in red cells (7) and because some proteins may already be partially or fully methylated.

In the experiments performed here, newly outdated human red blood cells were used. However, control experiments with freshly drawn human erythrocytes gave identical results.

Identification of Polypeptides Methylated in Vitro

Selective Extractions with Lithium Diodosalicylate and Triton X-100—To more precisely identify the radioactive polypeptides which had apparent M_r = 96,000 and 44,000 in Fig. 1, [3H]methylated membranes were extracted with either the chaotropic agent lithium diiodosalicylate or the mild nonionic detergent Triton X-100. Lithium diiodosalicylate has been shown to dissociate "extrinsic" proteins from the red cell membrane (17), while Triton X-100 has been shown to bind to and solubilize "intrinsc" red cell proteins (16, 24). In each case, the polypeptides remaining attached to the membranes and those which were dissociated were applied in parallel to dodecyl sulfate polyacrylamide gels and were analyzed as in Fig. 1.

Fig. 2 shows the result of extracting [3H]methylated membranes with 40 mM lithium diiodosalicylate at pH 6.8. As Steck and Yu have reported (17), bands 4, 5, and 6 were dissociated from the membranes by this treatment, while band 3 and the major sialoglycoprotein were not. Bands 1 and 2 are poorly resolved in this gel system, but are reported to be
Aliquots (5 μl) of [3H]methylated membranes prepared as described under "Experimental Procedures" or control membranes prepared by incubating an equivalent amount of purified membranes with 3.6 μM S-adenosyl-L-[3H]-methylmethionine in the absence of any cytosol were hydrolyzed for 4.5 h at 22°C in 100 μl of 1 M Tris-HCl at pH 9.0. Proteins were then precipitated with 1.5 ml of 20% trichloroacetic acid and the pellet, after a 9000 × g centrifugation for 4 min, was dissolved in 3% Protosol in Econofluor and counted after 24 h at 22°C for base-stable radioactivity. The total amount of protein-associated radioactivity was determined by following the procedure above but adding the trichloroacetic acid before the pH 9.0 buffer and incubating the mixture at 22°C for 2 h before centrifugation. The number of methyl groups transferred was determined from the radioactivity using an internal standard quench control and assuming that there was no dilution of isotope from endogenous adenosylmethionine. Base-labile incorporation was determined by subtracting base-stable incorporation from the total incorporation.

| Membranes                  | Methyl groups incorporated from S-adenosylmethionine into membranes | Total | Base-labile | Base-stable |
|----------------------------|---------------------------------------------------------------------|-------|-------------|-------------|
| + S-Adenosylmethionine     | 10.2                                                                | 6.0   | 4.2         |
| + S-Adenosylmethionine®    | 88.3                                                                | 84.7  | 3.6         |
| + Cytosolic fraction       |                                                                     |       |             |

* Standard lysed packed red cell preparation.

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**Fig. 2. Lithium diiodosalicylate extraction of [3H]methylated red blood cell membrane proteins and separation by dodecyl sulfate gel electrophoresis.** Top panel, 10% polyacrylamide gel electrophoresis of the supernatant fraction after treating membranes (44 μg of protein, 4.2 pmol of [3H]methyl groups) with 40 mM lithium diiodosalicylate at pH 6.8. The total amount of protein applied to the gel was 17 μg; the total number of [3H]methyl groups was 0.2 pmol. Bottom panel, gel electrophoresis of membrane pellet after the above extraction. The total amount of protein applied was 14 μg; the total number of [3H]methyl groups was 4.3 pmol. Each sample was applied to duplicate gels; one gel was stained with Coomassie blue and counted for total radioactivity, the other was stained with periodic acid-Schiff (PAS) reagent for carbohydrate. No PAS-positive bands were visible in gels containing the extract; the position of the major PAS band in the pellet is marked.

**Fig. 3. Triton X-100 extraction of [3H]methylated red blood cell membrane proteins and separation by dodecyl sulfate gel electrophoresis.** Top panel, 10% polyacrylamide gel electrophoresis of the supernatant fraction obtained by treating membranes (44 μg of protein, 4.2 pmol of [3H]methyl groups) with 0.2% Triton X-100. The total amount of protein applied was 14 μg; the total amount of [3H]methyl groups was 2.9 pmol. Bottom panel, gel electrophoresis of the membrane pellet after the extraction above. The total amount of protein applied was 31 μg; the total amount of [3H]methyl groups was 1.9 pmol. Gels were stained for protein with Coomassie blue and for carbohydrate with periodic acid-Schiff (PAS) reagent. No PAS-positive bands were visible in the gel containing the 0.2% Triton X-100 residue; the position of the major PAS-positive band in the extract is marked.
Protein Methylation in Erythrocyte Membranes

The Coomassie-stained gel in Fig. 4 indicates the absence of intact band 3 and the generation of the 59,000-dalton NH₂-terminal fragment of this polypeptide. The membrane-bound 35,000-dalton fragment is not clearly identifiable in these gels. The pattern of membrane-bound [3H]methyl radioactivity in the polypeptides of chymotrypsin-treated intact cells shows decreased radioactivity in the 96,000-dalton region and the appearance of a radioactive 59,000-dalton fragment. Periodic acid-Schiff staining of a similar gel showed no bands in the 70,000-dalton region (data not shown) and no radioactivity was incorporated into polypeptides of this molecular weight. A membrane-bound radioactive fragment of 35,000 daltons is also generated by chymotrypsin treatment. This polypeptide may be the COOH-terminal fragment of band 3, or it may be derived from other proteins.

The results presented thus far strongly indicate that both the major component of band 3 (the anion transport protein) and the major sialoglycoprotein are methylated in this system. At least one site of methylolation in the band 3 protein appears to be in the 59,000-dalton NH₂-terminal region which spans the membrane (see Fig. 5); the site of methylolation of the sialoglycoprotein is not known.

The chymotrypsin digestion also suggests that a minor polypeptide which co-migrates with the major band 3 polypeptide may be methylated. A substantial amount of radioactivity was incorporated into a polypeptide of Mᵦ = 96,000 after chymotrypsin digestion, even though very little of the Coomassie-staining protein remains.

Localization of a Methylated Site on the Major Band 3 Polypeptide—The chemistry of the major band 3 polypeptide has been studied in detail and the sites of cleavage by trypsin and chymotrypsin are known (29). In order to further investigate the location of the methylated site(s) in the 59,000-dalton NH₂-terminal region of band 3, [3H]methylated unsealed membranes were treated with trypsin. This digestion cleaves band 3 at two points (Fig. 5), yielding 23,000- and 20,000-dalton soluble NH₂-terminal fragments and a membrane-bound 55,000-dalton COOH-terminal fragment. The 23,000-dalton fragment contains the blocked NH₂ terminus (30, 31). As Fig. 6 shows, no radioactivity was found in soluble polypeptides after trypsin digestion, but a new membrane-bound methylated species at ~68,000 daltons was produced, which is probably the COOH-terminal fragment of band 3. This 68,000-dalton fragment contained essentially all of the radioactivity previously found in the intact band 3 molecule. This indicates that a site of methylation in the 59,000-dalton NH₂-terminal region of band 3 is in or near the 19,000-dalton fragment that spans the membrane (Fig. 5).

The digestion of unsealed [3H]methylated membranes by chymotrypsin generates a soluble 43,000-dalton NH₂-terminal fragment and a membrane-bound 19,000-dalton fragment.

The control pattern of radioactivity was similar to that shown in Fig. 1, but two radioactive polypeptides were distinguishable in the 27,000-dalton region (at 27,500 and 23,500 daltons), a major radioactive polypeptide was found at 70,000 daltons, and only a small amount of radioactivity was found at 41,500 daltons. Because periodic acid-Schiff staining revealed that the major sialoglycoprotein also migrated with an apparent Mᵦ = 70,000, it is likely that under these conditions (7.5% acrylamide gels), this glycoprotein has aggregated to a dimer. Thus, it is possible that the radioactivity at 41,500 daltons in these gels represents glycoporin monomer and/or other methylated polypeptides, while the radioactivity at 70,000 daltons represents the glycoporin dimer. Such changes in the position of the major glycoprotein have been well documented in other gel systems (25–27); it is not clear to us why the extent of dimerization under our conditions is variable.

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Protein Methylation in Erythrocyte Membranes

3072 Protein Methylation in Erythrocyte Membranes and aliquots of the soluble and membrane fractions obtained by centrifugation at 100,000 × g for 10 min corresponding to 100 μg of membrane protein were electrophoresed and fluorographed as above. No radioactivity was detected in the soluble fraction. Right, digestion of control membranes with 16 μg/ml of trypsin for 1 h at 0°C. Aliquots of the total digest and fractions of soluble and membrane fractions were prepared for fluorography as above. Again, no radioactivity was detected in the soluble fraction.

from the 59,000-dalton NH₂-terminal region of band 3 (18, 32) (Fig. 5). As Fig. 6 shows, no radioactivity was associated with the soluble cytoplasmic NH₂-terminal 43,000-dalton chymotryptic fragment or with the 19,000-dalton fragment, although both were clearly visible when the gels were stained with Coomassie (data not shown).

In order to determine whether the absence of radioactivity in the polypeptide fragments of band 3 after chymotryptic digestion was due to hydrolysis of the radioactive methyl groups or to the production of small peptides, the digestion mixture was applied to a column of Sephadex G-15 in 0.1 M acetic acid. Over 40% of the applied radioactivity was recovered in peptides smaller than hexaglycine and methanol accounted for less than 35% of the radioactivity (data not shown). Although the recovered peptides could be derived from polypeptides other than band 3, these results suggest that a site of methylation on band 3 is on a small peptide product of chymotryptic cleavage. A site for this peptide consistent with all of the data presented is at the junction of the 43,000-dalton NH₂-terminal fragment and the 19,000-dalton membrane-bound fragment (Fig. 5).

Characterization of Residues Methylated in Red Cell Membranes

Rate of Hydrolysis of [³H]Methylated Membrane Proteins in Base—In order to determine whether the overall rates of hydrolysis of the [³H]-methyl groups incorporated into red blood cell membrane proteins were consistent with the methylation of aspartic (8) or glutamic acid residues, the rates of hydrolysis of [³H]-methylated groups were compared with those of model compounds. Fig. 7 shows the fraction of radioactivity remaining as a function of time at pH 7.55 at 37°C for [³H]-methylated membranes prepared as in Fig. 4 from intact and trypsin-treated cells. The data in each case are consistent with the presence of linkages with at least three rates of hydrolysis ranging from a t₁/₂ of 3 min to one of 360 min. The half-lives of the three groups of alkali-labile linkages in methylated membranes from trypsin-treated cells are presented as a function of pH in Fig. 8 (left panel).

Comparison of Rates of Hydrolysis of Methylated Membranes in Mild Alkali with Those of Small Molecule Methyl Esters—As Fig. 8 shows, the rate of hydrolysis of membrane methyl groups was from one to three orders of magnitude faster in the pH range of 6.5–10.5 than aspartic acid β-methyl ester, the N-benzoyl derivatives of aspartic acid β-methyl ester and glutamic acid γ-methyl ester, and methyl propionate. The rates of hydrolysis of N-benzoyl-L-aspartyl β-methyl ester glycylamide, which has its α-amino and α-carboxyl groups in peptide linkages, however, were very similar to those of the bulk of the membrane methyl groups.

FIG. 6. Proteolysis of [³H]methylated red cell membranes. Left, fluorograph of a 10% polyacrylamide dodecyl sulfate gel of [³H]methylated membranes prepared by incubating packed, lysed red cells with S-adenosyl-l-[³H-methyl]methionine (20 μg of protein applied). Molecular weights of the prominent bands given are in kilodaltons. Center, digestion of control membranes with 0.2 mg/ml of chymotrypsin at 25°C for 2 h as described under “Experimental Procedures.” An aliquot containing 16 μg of protein of the total digest and aliquots of the soluble and membrane fractions obtained by centrifugation at 100,000 × g for 10 min corresponding to 100 μg of membrane protein were electrophoresed and fluorographed as above. No radioactivity was detected in the soluble fraction. Right, digestion of control membranes with 16 μg/ml of trypsin for 1 h at 0°C. Aliquots of the total digest and fractions of soluble and membrane fractions were prepared for fluorography as above. Again, no radioactivity was detected in the soluble fraction.

FIG. 7. Rate of hydrolysis at neutral pH of [³H]methylated membranes prepared from red blood cells and trypsin-treated red blood cells. Top panel, rate of hydrolysis of in vitro-labeled, [³H]methylated erythrocyte membranes at pH 7.55 and 37°C. Data were collected as described under “Experimental Procedures.” The smooth curve represents the exponential decay of three classes of sites with half-times of hydrolysis of 3, 30, and 360 min and proportions of 0.2, 0.3, and 0.5, respectively. Bottom panel, rate of hydrolysis of [³H]methylated membranes prepared from trypsin-treated cells (cf. Fig. 4). The smooth line was calculated for three groups with half-times of hydrolysis of 3, 30, and 190 min and proportions of 0.2, 0.3, and 0.5, respectively.
Fig. 8. Comparison of the rates of hydrolysis of [3H]methylated membranes from trypsin-treated red blood cells with those of model compounds. Red cell membranes were hydrolyzed in buffers of given pH at 37°C as in Fig. 7. Red cell membrane data are shown for the class of most rapid hydrolysis (O-O), intermediate hydrolysis (□□□□□), and slowest hydrolysis (△-△). Data for small molecule methyl esters were obtained as described under "Experimental Procedures."
The rates of hydrolysis of both free L-glutamic acid γ-methyl ester and its derivative, N-benzoyl-L-glutamyl γ-methyl ester glycylamide, were similar to those of the membrane methyl groups with the slowest rates of hydrolysis.

In contrast, N-acetyl-L-leucine methyl ester, chosen to simulate a COOH-terminal methylated amino acid, had a hydrolysis rate considerably slower than those of the membrane methyl groups.

Comparison of Rates of Hydrolysis of Methylated Membranes in 6 M HCl with those of Small Molecule Methyl Esters—The time course of hydrolysis in 6 M HCl of [3H]-methylated membranes prepared from trypsin-treated cells also indicates the presence of linkages with at least three rates of hydrolysis (ranging from a t1/2 of 3 min to one of 320 min) (Fig. 9). Table II gives data for the rates of hydrolysis of small molecule methyl esters in 6 M HCl. These rates (expressed in terms of half-lives) vary from 5 min for methyl propionate to 470 min for L-leucine methyl ester and are consistent with the half-lives of 3-360 min measured for the red cell membrane methyl groups (Figs. 8 and 9).

**Fig. 9.** Rate of hydrolysis in 6 M HCl of [3H]methylated membranes prepared from trypsin-treated red blood cells. For experimental details, see Fig. 7 and "Experimental Procedures." The smooth curve represents the hydrolysis of three species with half-lives of 3, 60, and 320 min and proportions of 0.25, 0.68, and 0.07, respectively.

### TABLE II

| Ester                        | \( t_{1/2} \) (min) |
|------------------------------|---------------------|
| L-Aspartic acid β-methyl ester | 108                 |
| N-Benzoyl-L-aspartic acid β-methyl ester | 36       |
| L-Glutamic acid γ-methyl ester | 1.5               |
| N-Benzoyl-L-glutamic acid γ-methyl ester | 10.5   |
| L-Leucine methyl ester       | 470                 |
| Methyl propanoate            | 5                   |

* Determined from a semilogarithmic plot of ester remaining versus time. Ester concentration was determined by the hydroxylamine/ferric chloride assay described under "Experimental Procedures."

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**DISCUSSION**

*S-Adenosyl-L-Methionine-dependent Protein Methylation in Lysed Human Erythrocytes—*Kim (4) has described an enzyme from the cytosol of human red cells which catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to exogenous proteins such as ovalbumin and ribonuclease. The methyl group was found to be labile under mildly basic conditions. O'Dea et al. (33) showed that the membrane fraction of erythrocytes contained sites which could be methylated by this enzyme, and that no methyl-accepting proteins were detectable in the cytosol. Galletti et al. (5, 6) have incubated purified methyltransferases with rat and human erythrocyte membranes in order to characterize the methyl-accepting proteins.

We have investigated these methylation reactions under *in vitro* conditions that paralleled as nearly as possible the conditions in intact red cells. Because biological membranes are generally impermeable to S-adenosylmethionine, it was necessary to disrupt the red cell membrane to allow the S-adenosyl-L-[3H-methyl]methionine access to the cytosolic enzyme. This was done in the present study by a freeze-thaw lysis. To keep the concentration of cytoplasmic components, including the methyltransferase(s), methyltransferases, activating molecules, and inhibiting molecules as close to physiological levels as possible, we employed packed red cells. These cells have a very low extracellular volume and were only minimally diluted with the addition of isotopic S-adenosyl-L-methionine.

**Identification of Polypeptides Methylated in Human Erythrocyte Membranes in Vitro**—The identification of potential substrates for the erythrocyte protein methyltransferase is a first step in elucidating the role of this enzymatic system in eucaryotic cells. The human erythrocyte proteins are well characterized and the functions of several have been identified. The bulk of the band 3 polypeptide is homogeneous with respect to its amino acid sequence and other properties (29), spans the membrane, and functions in the transport of anions in intact cells (34). The major sialoglycoprotein (PAS-1, glycoporphin) also spans the membrane (35); its function is as yet unclear. The present work shows that both of these polypeptides are methylated in a broken cell system. In the band 3 polypeptide, at least one site of methylation appears to be at the junction between the cytoplasmic NH2-terminal 43,000-dalton region and the 19,000-dalton internal hydrophobic segment. We have not yet determined whether any other sites of methylation on band 3 or those on the major sialoglycoprotein are internal or external to the erythrocyte and, hence, whether these sites are in fact available to the cytoplasmic protein methyltransferase. As protein methyltransferase activity is very low in the blood plasma (36), external red blood cell sites of methylation are likely to be artifactual.

We have also shown that two minor polypeptide components of human red cell membranes are methylated in this *in vitro* system. One of these is a peptide that co-migrates on dodecyl sulfate gel electrophoresis with the band 3 anion transport protein (96,000 daltons), but which can be distinguished because it is not removed by extracellular chymotrypsin treatment. Two others migrate at polypeptide \( M = 23,000 \) and 27,000 and appear to be intrinsic membrane proteins as they can be extracted with Triton X-100, but not with lithium diiodosalicylate.

No evidence has been found in this broken cell system for methylation of bands 1 and 2 (spectrin), band 2.1 (ankyrin), band 4, zone 4.5 (which contains the glucose transport protein), band 5 (actin), or band 6 (glyceraldehyde-3-phosphate dehydrogenase). The lack of methylation of these proteins...
shows that there is specificity in the protein methyltransferase reaction(s).

Galletti et al. (6) have concluded that the major sialoglycoprotein (glycophorin A) and band (zone) 4.5 are the predominant methyl acceptors in human erythrocyte membranes when methylation reactions are catalyzed by a purified methyldonorase. The results of the present study, in which red blood cell membranes were methylated in a broken cell system with cytosolic components at near-physiological levels, also indicate that the major sialoglycoprotein is a methyl group acceptor. However, zone 4.5 was not found to be methylated in our study. Furthermore, we present evidence here that the primary component of band 3, known to be involved in anion transport, is also a methyl acceptor.

Characterization of the Chemical Site of Methylation—It has recently been shown that at least some of the sites of base-labile methylation in human red blood cell membrane proteins are at aspartic acid residues (8). The rate of hydrolysis of the bulk of the base-labile methyl groups, however, has been found to be much more rapid than that of aspartic acid β-methyl ester (Figs. 7 and 8) (Refs. 5, 37, 38). In order to determine whether these rapidly hydrolyzable membrane methyl groups might also be carboxylic acid esters, we compared the rates of hydrolysis of the membrane methyl groups with those of aspartic acid β-methyl ester and glutamic acid γ-methyl ester and their derivatives in which the α-carboxyl and α-amino groups are involved in peptide bonds.

We find that glutamic acid γ-methyl ester and its derivative N-benzoylglycylglycine have base hydrolysis rates similar to those of the slowest group of membrane methyl groups. Additionally, while aspartic acid β-methyl ester has hydrolysis rates considerably slower than those of the membrane methyl groups, N-benzoylaspartyl β-methyl ester glycylamide has rates of hydrolysis similar to those of the bulk of the membrane methyl groups. Thus, the rates of hydrolysis of the methylated membrane sites are consistent with the methylation of internal aspartic acid and glutamic acid residues, although direct evidence has only been found for aspartyl residues (8).

One explanation for the rapid hydrolysis of internal aspartic acid residues is suggested by the studies of Bernhard et al. (39). They found that the rate of base-catalyzed hydrolysis of β-benzyl esters of N-carbobenzyloxyaspartyl peptides was up to 10-fold greater than that for benzyl propionate. They demonstrated that the rapid reaction proceeded via an imine intermediate involving the amide nitrogen of the peptide bond on the carboxyl side of the aspartyl residue. Such a large rate enhancement was not found for similar glutamyl peptides (40, 41).

Although it is likely that demethylating enzymes play a role in the hydrolysis of eucaryotic protein-bound methyl esters, it is possible that a rapid spontaneous hydrolysis via an imine intermediate may also be important. If this is the case, such an intermediate can be potentially hydrolyzed to give a free α-carboxyl group and a peptide linkage of the side chain carboxyl group to the rest of the polypeptide chain. Whether this occurs in vivo has not yet been established. Additionally, the rate of hydrolysis of the methyl esters would be expected to be highly dependent upon the conformation of the protein at the methylated site.

Relation of In Vitro Methylation to Physiological Methyltransferase Reactions in Intact Cells—Because the methylation reactions studied here occurred in a broken cell system, it is possible that qualitative and quantitative differences from the results obtained here occur in in vivo methylation. In the broken cell system, cytosolic methyltransferases have access to polypeptide residues on the exterior membrane surface. Although we have tentatively shown that at least one site of methylation of band 3 is at the internal surface of the membrane, it is possible that some of the methylated sites observed in this study and previously (5, 6) are not methylated in intact cells. In a preliminary study, Kim et al. (7) have tentatively shown that the pattern of methylation of red cell membrane proteins in vivo is similar to the in vitro pattern. If the same residues in each polypeptide are methylated in vivo and in vitro, then the broken cell system will be an appropriate model for studying the physiological role of this covalent modification.

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Note Added in Proof—We have recently shown that although band 3 is in fact methylated in intact cells, the major sialoglycoprotein is not (C. Freitag and S. Clarke, manuscript in preparation).

REFERENCES

1. Springer, M. S., Goy, M. F., and Adler, J. (1979) Nature 280, 279–284
2. Koshland, D. E., Jr. (1980) Bacterial Chemotaxis as A Model Behavioral System, Raven Press, New York
3. Paik, W. K., and Kim, S. (1980) Protein Methylation pp. 202–231, John Wiley, New York
4. Kim, S. (1974) Arch. Biochem. Biophys. 161, 652–657
5. Galletti, P., Paik, W. K., and Kim, S. (1978) Biochemistry 17, 4272–4276
6. Galletti, P., Paik, W. K., and Kim, S. (1979) Eur. J. Biochem. 97, 221–227
7. Kin, S., Galletti, P., and Paik, W. K. (1980) J. Biol. Chem. 255, 338–341
8. Janson, C. A., and Clarke, S. (1980) J. Biol. Chem. 255, 11640–11643
9. Steck, T. L., and Kant, J. A. (1974) Methods Enzymol. 31, 172–180
10. Dodge, J. T., Mitchell, C., and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 199–207
11. Fairbanks, G., and Avruch, J. (1972) J. Supramol. Struct. 1, 66–75
12. Studier, F. W. (1973) J. Mol. Biol. 79, 237–258
13. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) Biochemistry 10, 2006–2017
14. Chamberlain, J. P. (1979) Anal. Biochem. 98, 132–135
15. Leggett Bailey, J. (1967) Techniques in Protein Chemistry p. 340, American Elsevier, New York
16. Yu, J., Fischman, D. A., and Steck, T. L. (1973) J. Supramol. Struct. 1, 231–248
17. Steck, T. L., and Yu, J. (1973) J. Supramol. Struct. 1, 239–252
18. Rao, A., and Reithmeier, R. A. F. (1979) J. Biol. Chem. 254, 6144–6150
19. Trickett, K. L. (1976) J. Biol. Chem. 251, 5115–5123
20. Hestrin, S. (1949) J. Biol. Chem. 180, 249–261
21. Vogel, A. I. (1965) A Textbook of Practical Organic Chemistry, 3rd Ed, p. 436, John Wiley, New York
22. Hoare, D. G., and Koshland, D. E., Jr. (1967) J. Biol. Chem. 242, 2447–2453
23. Hirata, F., and Axelrod, J. (1978) Nature 275, 219–220
24. Clarke, S. (1975) J. Biol. Chem. 250, 5459–5469
25. Marton, L. S. G., and Garvin, J. E. (1973) Biochem. Biophys. Res. Commun. 52, 1457–1462
26. Furthmayr, J., and Marchesi, V. T. (1976) Biochemistry 15, 1137–1144
27. Silverberg, M., Furthmayr, H., and Marchesi, V. T. (1976) Biochemistry 15, 1448–1454
28. Triplett, R. B., and Carraway, K. L. (1972) Biochemistry 11, 2907–2903
29. Steck, T. L. (1978) J. Supramol. Struct. 8, 311–324
30. Steck, T. L., Koziarz, J. J., Singh, M. K., Reddy, G., and Kohler, H. (1978) Biochemistry 17, 1216–1222
31. Drickamer, L. K. (1978) J. Biol. Chem. 253, 7242-7248
32. Steck, T. L., Hames, B., and Strapazon, E. (1976) Biochemistry 15, 1154-1161
33. O'Dea, R. P., Viveros, O. H., Acheson, A., Gorman, C., and Axelrod, J. (1978) Biochem. Pharmacol. 27, 679-684
34. Cabanchik, Z. I., Knauf, P. A., and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 289-302
35. Marchesi, V. T. (1979) Semin. Hematol. 16, 3-20
36. Kim, S., Wasserman, L., Lew, B., and Paik, W. K. (1975) J. Neurochem. 24, 623-629
37. Kim, S., and Paik, W. K. (1976) Experientia 32, 982-984
38. Diliberto, E. J., Jr., and Axelrod, J. (1976) J. Neurochem. 26, 1159-1165
39. Bernhard, S. A., Berger, A., Carter, J. H., Katchalski, E., Sela, M., and Shalitin, Y. (1962) J. Amer. Chem. Soc. 84, 2421-2434
40. Fosker, A. P., Hasson, R. W., and Law, H. D. (1963) Chem. Ind. 569-570
41. Battersby, A. R., and Robinson, J. C. (1955) J. Chem. Soc. 259-265