Reversible Methylation of Cytoskeletal and Membrane Proteins in Intact Human Erythrocytes*

(Received for publication, December 15, 1980, and in revised form, February 12, 1981)

Catherine Freitag‡ and Steven Clarke§

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

Membrane protein methylation was studied in intact human erythrocytes. Erythrocytes were incubated with physiological concentrations of \( \text{L-[methyl-}^3\text{H}] \text{methionine} \) and incorporated 35 pmol of methyl groups into membrane components/mg of membrane protein in a 2.5-h incubation at 37 °C. At least 90% of the total groups (12,500 methyl groups/cell) were incorporated into polypeptides via linkages which were labile to 1 N NaOH.

Major methylated membrane polypeptides were identified based on the comigration of radioactivity with Coomassie blue- and periodic acid-Schiff-staining species in pH 2.4 dodecyl sulfate gel electrophoresis, as well as by the distribution of radolabel and protein following selective proteolysis and membrane extraction procedures.

Methylated species identified in this way include the cytoskeletal polypeptides band 2.1 (ankyrin) and band 4.1, as well as the band 3 anion transport protein. Other methylated species include an intrinsic polypeptide migrating with band 3 and other sensitive to external chymotrypsin digestion, a glycoprotein showing variable migration in this gel system (40,000–55,000 daltons), an intrinsic polypeptide at about 30,000 daltons, and an extrinsic species of about 17,000 daltons. A small amount of radioactivity comigrated with the band 4.5 region. Bands 1, 2, 4.2, 5, 6, 7, and the major sialoglycoprotein were not methylated in this system. All of the methylated species exhibited turnover in vivo, and the time taken to reach 50% demethylation for each species ranged from less than 2 to 29 h.

Enzymes which catalyze the S-adenosylmethionine-dependent incorporation of base-labile methyl groups into proteins have been described from many sources (for a review see Ref. 1). In enteric bacteria, the enzymatic formation and hydrolysis of glutamic acid \( \gamma \)-methyl esters on a class of membrane proteins has been shown to play an integral role in the chemotaxis of these procaryotes (2, 3). Despite the apparent ubiquitous occurrence of enzymes in eucaryotic cells which catalyze similar methyltransferase reactions, the chemical basis and the physiological significance of this type of post-translational covalent modification remain unclear.

The human red blood cell provides a well characterized system for studying S-adenosylmethionine-dependent protein carboxyl methylation. Many of the major polypeptides in both the membrane and cytosolic fraction have been functionally identified and chemically characterized (for a review see Ref. 4). Thus, \( \kappa \) may be possible in the red cell to identify methyl acceptor species with functional proteins and characterize the physiological consequences of this modification.

Initial investigations of protein methylation in erythrocytes have focused on in vitro studies. A protein methyltransferase has been identified (5) and has been used to catalyze the incorporation of methyl groups from S-adenosyl-L-[methyl-\( ^3\)H]methionine into isolated rat (6) and human (7, 8) erythrocyte membranes. Analysis of the methyl acceptor species by dodecyl sulfate gel electrophoresis revealed the specific methylation of several membrane polypeptides. In an attempt to perform these reactions under more physiological conditions, we have recently employed a broken cell system and have shown that major methyl acceptor species in human red cells are the band 3 anion transport component and the major sialoglycoprotein (9). It has also been possible to demonstrate the formation of \( \beta \)-methyl ester linkages in aspartyl residues in this preparation (10).

It is clear, however, that the physiologically significant methylation reactions are those that occur in intact cells. By incubating whole red cells with L-[methyl-\( ^3\)H]methionine, Kim et al. (11) have made a tentative identification of the major methylated species as the major sialoglycoprotein and band 4.5. To more precisely identify the methyl-accepting polypeptides, we have utilized in this study a gel system operating at pH 2.4 to reduce nonenzymatic hydrolysis, and have used selective proteolysis and extractions to make definite assignment of the methylated species. We confirm here the occurrence of in vivo protein methylation, but report that the major methylated species include at least two cytoskeletal proteins and the band 3 anion transport protein, rather than the major sialoglycoprotein and band 4.5, as previously reported (11).

MATERIALS AND METHODS

Red Cell Isolation—Heparinized blood, obtained by venipuncture from a volunteer donor, was centrifuged at 1000 \( \times g \) for 10 min at 20 °C. The plasma and buffy coat were removed and the red cells were resuspended in 20–30 volumes of ice-cold phosphate-buffered isotonic saline (150 mM NaCl, 5 mM sodium phosphate, pH 7.0) and pelleted (3000 \( \times g \) for 10 min at 2 °C). The resuspension step was repeated three times; the final pellet was resuspended in phosphate-buffered saline containing 0.167 M glucose to a protein concentration of 92 mg/mL.

Preparation of in Vivo Methylated Membrane Proteins—An aliquot of L-[methyl-\( ^3\)H]methionine (80 Ci/mmol, in ethanol:water 7:3, from New England Nuclear) was dried under a gentle stream of purified nitrogen gas at 20 °C. To the dried methionine, enough of the red cell suspension (92 mg/mL) described above was added to give a final L-[methyl-\( ^3\)H]methionine concentration of 12.7 to 40 \( \mu \)M. After a 2.5-h incubation at 37 °C on a shaking water bath, 20–30 volumes of ice-cold phosphate-buffered saline were added and the cells were

* This research was supported by United States Public Health Service Grants GM-26020 and RR-7009 (Biomedical Research Support Grant to H. Lewis) 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.

‡ University of California President’s Undergraduate Fellow.

§ To whom correspondence should be addressed.
**Methylation of Red Cell Cytoskeletal and Membrane Proteins**

Methylated membranes were prepared from the red cell pellet by hypotonic lysis as described by Dodge et al. (12), modified by Terwilliger and Clarke (9). Briefly, cells were suspended in 30 volumes of 5 mM sodium phosphate, pH 8.0, for 1 min at 2 °C. The pH was then lowered to 6.8 by the addition of sodium citrate buffer and the membrane pellets were washed one to three additional times as described in the figure legends. Approximately 0.5 ml of membranes was obtained from 1 ml of packed cells at a protein concentration from 3 mg/ml to 5 mg/ml in 4.3 mM sodium phosphate, 5.3 mM sodium citrate, pH 6.8.

Some electrophoreses were performed by dodecyl sulfate gel electrophoresis—Electrophoresis was performed in a pH 8.4 buffer system developed by Fairbanks and Ahruch (13).

Samples of membranes were assayed for protein by a modification of the Lowry protocol, using bovine serum albumin as a standard (14), and appropriate amounts were mixed (12, v/v) with 4% dodecyl sulfate, 24% glycerol, 75 mM sodium phosphate, 0.06% Pyronin Y, 286 mM β-mercaptoethanol, pH 2.4, and were heated 3 min at 100 °C. Electrophoresis was performed on 1.5-mm thick slab gels in an apparatus similar to that described by Studier (15). The electrophoresis was started at 100 V, and after all tracking dye entered the separating gel, the voltage was increased to 500 V. The tracker dye reached the bottom of the separating gel (10 cm). Gels contained acrylamide and N,N'-methylenebisacrylamide in a weight ratio of 29:1.

Molecular weight assignments were made on the basis of marker proteins electrophoresed in parallel lanes and included myosin (Mr = 200,000), β-galactosidase (117,000), phosphorylase b (92,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (21,500), and lysozyme (14,300).

Detection of Radioactivity in Membrane Fractions and in Individual Polypeptides—Total incorporation of radioactivity in trichloroacetic acid-insoluble material was determined by precipitating 10 ml of membranes with 1.5 ml of 15% trichloroacetic acid (v/v) in a 1.7-ml microfuge tube, pelleting the proteins (9000 x g for 4 min), and counting the pellet after 18 h at 30 °C in 1.5 ml of 3% NCS tissue solubilizer (Amersham) in OCS counting fluid (Amersham). Total incorporation of radioactivity into protein in microfuge pellets separated by dodecyl sulfate electrophoresis was measured by fluorochemistry of Coomassie blue-stained gels using a Unisalt salicylate as described by Chamberlain (17). Gels were enclosed in cellophane-backed Kodak X-Omat R film for 1 to 3 weeks at ~70 °C. Radioactivity in individual bands was quantitated by film densitometry in a Joyce-Loebel instrument. Alternatively, 1-mm gel slices were counted directly after incubation at 37 °C for 18 h in 4 ml of 3% NCS tissue solubilizer in OCS counting fluid. In each method listed above, counts were corrected for quenching by using an internal standard method.

Base labile radioactivity was assayed by two methods. Samples of membranes (10 ml) in 1.7-ml microfuge tubes were treated with 30 ml of 1 N NaOH, and after 1 min (30 °C), 1.5 ml of 15% trichloroacetic acid were added. Proteins were pelleted (9000 x g x 4 for 1 ml) and an aliquot of the supernatant was counted for radioactivity in 10 ml of Handifluor (Mallinckrodt). This number, after correcting for and the volume counted, was considered to represent base-stable counts. The pellet remaining in the microfuge tube was counted using 3% NCS in OCS-solubilized fluid. In this number, all the appropriate corrections, was considered to represent base-stable radioactivity.

Base-labile, volatile radioactivity was measured by the hydrolysis and microdistillation procedure of Stock and Koshland. A 10-ml sample of methylated membranes was incubated with 30 ml of 1 N NaOH, followed by 1 ml of methanol and heating at 80 °C. The volatile contents were collected in Handifluor with an additional 1-ml rinse of methanol. The number of counts in the vial was taken to represent the number of base-labile volatile counts associated with the membrane sample. The number of counts remaining in the test tube was taken to represent base stable incorporation. Corrections were made for quenching and for the efficiency of the distillation. Individual gel slices were also assayed in this way, except that 5-10 min elapsed between the addition of 1 N NaOH and the addition of 1 ml of methanol to allow the gel slices to swell.

**Detection of Radioactivity in Lipid—Red blood cells were incubated for 2.5 h at 37 °C in 12.7 μM [methyl-3H]methionine and after all tracking dye entered the separating gel, the voltage was increased to 500 V. The tracker dye reached the bottom of the separating gel (10 cm). Gels contained acrylamide and N,N'-methylenebisacrylamide in a weight ratio of 29:1.**

**RESULTS**

**Membrane Protein Methylation in Intact Human Erythrocytes**

Intact red blood cells were incubated with physiological concentrations of [methyl-3H]methionine (20) in phosphate-buffered isotonic saline containing glucose. This amino acid is rapidly transported across the membrane (21) and is then converted to the methyl group donor S-adenosyl-L-[methyl-3H]methionine by an endogenous activity in the cytosol of the cell (22).

Table I shows that a total incorporation of 35 pmol of methyl groups/mg of membrane protein occurs when erythrocytes are incubated for 2.5 h at 37 °C in 12.7 μM [methyl-3H]methionine. Of this total incorporation, approximately 90% of the labeled methyl groups in the membranes are linked to proteins via base-labile linkages (31.9 pmol of methyl groups/mg of membrane protein). This number is a minimum estimate of the radioactivity associated with polypeptides, since it accounts for only base-labile linkages. The remaining radioactivity is in either base-stable linkages of methyl groups to lipid, protein, or other unidentified membrane components. Approximately 1.4% of the labeled methyl groups in the membranes are linked to phospholipids or to other compounds extractable into chloroform/methanol (0.48 pmol of methyl groups/mg of membrane protein).

As shown in Fig. 1, no polyolipide of the red cell membrane appears to be methylated solely in base-stable linkages, because labeled methyl groups have been demonstrated for each of the labeled species. For example, an assay of similar gel slices for total counts (data not shown) gives a pattern of radioactivity which coincides exactly with that of the base-labile distribution of radioactivity illustrated in Fig. 1.

The bulk of the incorporation of [3H]-methyl groups is in polyolipides of Mr = 200,000, 98,000, 83,000, and 45,000. Minor

---

1. The abbreviation used is PAS, periodic acid-Schiff.
2. J. B. Stock and D. E. Koshland, Jr., manuscript in preparation.
The incorporation of \(^3\)H-methyl groups into red cell membrane proteins was determined for cells incubated for 2.5 h at 37 °C in a total concentration of either 12.7 or 40 \(\mu\)M L-[methyl-\(^3\)H]methionine as described under "Materials and Methods." The total number of methyl groups incorporated and the number of methyl groups incorporated into base-stable and base-labile linkages were determined by two methods. Samples after base hydrolysis were separated into fractions containing base-labile and base-stable radioactivity by either distillation or trichloroacetic acid precipitation as described under "Materials and Methods."

| Distillation assay | Trichloroacetic acid precipitation assay | Distillation assay | Trichloroacetic acid precipitation assay |
|-------------------|----------------------------------------|-------------------|----------------------------------------|
| 40 \(\mu\)M       | 12.7 \(\mu\)M                          | 40 \(\mu\)M       | 12.7 \(\mu\)M                          |
| \(^3\)H methyl groups/mg membrane protein | Methyl groups/cell |
| Total             | 35.1                                   | 35.3              | 13,700                                  | 13,800                                   |
| Base-stable       | 4.1                                    | 2.4               | 1,600                                    | 940                                      |
| Base-labile       | 31.9                                   | 33.0              | 12,500                                  | 12,900                                   |

- Average values of two determination of \(^3\)H-methylated membranes.
- Determined from analysis of 10-21 samples (48 \(\mu\)g of membrane protein) of \(^3\)H-methylated membranes.
- A minimum estimate based on the assumption that the pool of S-adenosylmethionine reaches the same specific activity as the [methyl-\(^3\)H]methionine. The latter specific activity was calculated by accounting for the dilution of isotope by an endogenous pool of 25 \(\mu\)M methionine (22). The number of moles of nonisotopically labeled methionine in the incubation (92 mg of red cell protein/ml of incubation) was calculated using the value of 320 mg of red cell protein/ml of packed cells (12).
- Calculated based on the red cell content of 6.5 \(\times\) 10\(^{-10}\) mg of membrane protein/cell (12).
- Determined by summing radioactivity in the lane of the dodecyl sulfate polyacrylamide gel shown in Fig. 1.

The radioactive species migrating at 32,500 daltons has a half-life of approximately 2 h, and the radioactivity comigrating with hemoglobin appears to completely turn over within the initial period of 4 h.

### Identification of Polypeptides Methylated in Vivo

#### Proteolytic Digestions of Intact Cells and Membranes—
Figs. 1 and 2 have shown the comigration of the 200,000-dalton methylated species with band 2.1, the 98,000-dalton methylated species with band 3, and the 83,000-dalton methylated species with band 4.1. Since the major sialoglycoprotein also comigrates with the latter two radiolabeled species (Fig. 3), selective proteolysis was employed to more precisely identify the methylated species.

Trypsin proteolytic digestion of intact red blood cells selectively cleaves the major sialoglycoprotein (PAS 1) into small fragments but does not affect the major polypeptides which are stained by Coomassie blue (23, 24). Fig 3 shows the results of a trypsin digestion of intact erythrocytes previously incubated with 20 \(\mu\)M L-[methyl-\(^3\)H]methionine. Neither the Coomassie blue-staining pattern nor the distribution of radioactivity of the trypsin-digested cells differ from the control; however, in the trypsin-digested cells there is no longer detectable periodic acid-Schiff staining of the major sialoglycoprotein. Since there is no loss of radioactivity in the 97,000- and 84,000-dalton polypeptides, we conclude that these methylated polypeptides cannot be attributed to the major sialoglycoprotein (PAS 1).

The peak of radioactivity migrating at 55,000 daltons on the 5% acrylamide gel in Fig. 3 is not sensitive to trypsin digestion in intact cells and comigrates with a minor periodic acid-methylated species with apparent \(M_t\) = 30,000 and 16,000 have also been detected. The peaks of radioactivity migrating at 200,000, 98,000, and 83,000 daltons comigrated with Coomassie blue-staining bands 2.1, 3, and 4, respectively. The radioactivity at 45,000 and 30,000 daltons does not comigrate with any specific Coomassie blue-staining bands.

The radioactivity in the peak at 16,000 daltons is not proportional to the amount of hemoglobin present in different preparations of membranes, suggesting that either hemoglobin is variably methylated or that this radioactive species is due to another polypeptide which comigrates with hemoglobin.

#### Turnover Rates of \(^3\)H-Methyl Groups in Erythrocyte Membrane Polypeptides

Intact red blood cells were pulsed with 12.7 \(\mu\)M L-[methyl-\(^3\)H]methionine for 2.5 h at which time excess nonsitopically labeled L-methionine was added. Fig. 2 shows the distribution and intensity of \(^3\)H-methylated membrane polypeptides present at the initiation of the chase, and at 4.8, and 23.5 h. In this gel (5% acrylamide), band 4 is resolved into bands 4.1 and 4.2, and the peak of radioactivity migrating at 45,000 daltons in Fig. 1 is now found at an estimated \(M_t\) of 55,000. Each methylated polypeptide was found to turn over at a characteristic rate. The peak of radioactivity comigrating with band 2.1 shows the slowest rate of turnover with a half-life of approximately 28 h. The peaks of radioactivity comigrating with band 3 and band 4.1 and the radioactive species migrating at 55,000 daltons have half-lives of 8, 3, and 2 h, respectively.
Methylation of Red Cell Cytoskeletal and Membrane Proteins

To test whether the methylated species migrating at about 97,000 daltons is the polypeptide of the band 3 anion transport protein (26), intact red cells were incubated with [methyl-3H]methionine and were then digested with α-chymotrypsin. The external digestion of red cells with α-chymotrypsin has been shown to cleave PAS 1 into small fragments and to cleave band 3 into two membrane-bound fragments of approximate Mr = 35,000 and 65,000 (26, 27). The Coomassie blue stain of polypeptides from chymotrypsin-treated cells in Fig. 4 shows the appearance of a new polypeptide at 64,000 daltons and the loss of the 97,000-dalton polypeptide band 3. The appearance of the 35,000-dalton fragment was not observed in this experiment.

The distribution of radiolabeled polypeptides of membranes prepared from chymotrypsin-digested cells is similar to the distribution of the control cells, except for the generation of a new methylated polypeptide which comigrates with the band 3 64,000-dalton fragment. Despite the fact that virtually all the Coomassie blue stain of band 3 has disappeared in the chymotrypsin-treated cells, there is still some radioactivity in this region. This indicates the existence of at least two methylated components (one chymotrypsin-insensitive and the band 3 polypeptide) migrating in the band 3 region. Chymo-

Schiff-staining component. The molecular weight calculated for this radiolabeled peak varies with the amount of acrylamide in the gel (cf. Fig. 1). The variable migrations of the PAS components relative to the Coomassie blue-stained com-

ponents have been described by Marton and Garvin (25).

FIG. 2. Reversibility of protein methylation in intact erythrocytes and turnover of methyl groups in a pulse-chase experiment. Red blood cells were pulsed with 12.7 μM L-[methyl-

3H]methionine at 37 °C. After 2.5 h, 20–30 volumes of ice-cold phosphate-buffered saline were added, cells were pelleted and were resuspended in a chase of 6 mM nonisotopically labeled methionine in phosphate-buffered saline containing 0.167% glucose at 37 °C. Aliquots were withdrawn from the incubation mixture at various times and membranes were prepared as described. Samples of membranes (40 μg of protein) were subjected to dodecyl sulfate gel electrophoresis (5% polyacrylamide gel). A densitometric trace of Coomassie blue stain (dashed line) is shown superimposed on a densitometric trace of a fluorograph of the same lane (cross-hatched area). Data are shown for samples taken at 0, 4, 8, and 23.5 h. An estimation of the percentage of methyl groups turned over was made by integrating individual peaks of radioactivity and comparing this number with the amount of total protein (determined by integration of a densitometric scan of the Coomassie blue stain).

FIG. 3. Proteolysis of red cell membrane proteins by digestion of intact cells with trypsin. Red blood cells were incubated in 20 μM L-[methyl-3H]methionine for 2.5 h at 37 °C, washed once, and incubated with and without trypsin (10 μg/ml) for 90 min as described under "Materials and Methods." Samples of proteolyzed and nonproteolyzed membranes (50 μg of protein) were subjected to dodecyl sulfate gel electrophoresis (5% polyacrylamide gel). A densitometric trace of the Coomassie blue stain, periodic acid-Schiff stain, and of the fluorograph are shown above by the dashed line, solid line, and cross-hatched area, respectively. Since the migration of the dye front and the patterns of radioactivity were identical in the lanes analyzed by PAS and Coomassie blue staining, the densitometric traces are shown above superimposed.
Methylation of Red Cell Cytoskeletal and Membrane Proteins

Membranes prepared from red blood cells which had incubated with L-[methyl-\(^{3}H\)]methionine were extracted with 40 mM lithium diiodosalicylate, and samples of the depleted membrane fraction and the supernatant were analyzed by dodecyl sulfate gel electrophoresis (Fig. 5). The membrane residue contains the Coomassie blue stain for band 3 and the radioactivity comigrating with band 3. The residue also contained the methylated periodic acid-Schiff-staining 43,000-dal-

-trypsin digestion also decreases the intensity of the 44,000-dalton radiolabeled species observed in the control.

In order to determine if the methylated species migrating at 200,000 daltons is actually band 2.1, we performed a chymotryptic digestion on spectrin-depleted inverted vesicles, prepared by the method of Bennett and Branton (28) from the chymotrypsin-digested intact cells in Fig. 4. Selective proteolysis of these vesicles by \(\alpha\)-chymotrypsin (29) was found to generate a soluble 72,000-dalton radioactive fragment with a concomitant disappearance of the radioactivity comigrating with band 2.1 (data not shown). The radioactivity comigrating with band 4.1 (see Fig. 4) was not detectable after this treatment; this is consistent with the results of Bennett showing that this polypeptide disappears after the chymotrypsin digestion (29).

Lithium diiodosalicylate extraction of membranes selectively releases the extrinsic membrane polypeptide bands 1, 2, 2.1, 4.1, 4.2, 5, and 6, while bands 3, 7, zone 4.5, and the glycoproteins are retained in the membrane fraction (30).

**Fig. 4.** Proteolysis of red cell membrane proteins by digestion of intact cells with \(\alpha\)-chymotrypsin. Red blood cells were incubated in 12.7 µM L-[methyl-\(^{3}H\)]methionine for 2.5 h at 37 °C, washed once, and incubated with or without \(\alpha\)-chymotrypsin (275 µg/ml) for 90 min as described under "Materials and Methods." Membranes (50 µg of protein) were prepared as described and were subjected to dodecyl sulfate gel electrophoresis (10% polyacrylamide gel). A densitometric trace of Coomassie blue stain is shown superimposed on a densitometric trace of fluorograph of the same lane.

**Fig. 5.** Selective solubilization of extrinsic membrane proteins by lithium diiodosalicylate. Red blood cells were incubated in 12.7 µM L-[methyl-\(^{3}H\)]methionine and membranes were prepared with 2 washes. One volume of membranes was mixed with 6 volumes of 40 mM lithium diiodosalicylate in 5 mM sodium phosphate, pH 6.8, and incubated at 0 °C for 30 min. Membranes were pelleted (27,000 X g for 20 min), washed once, and 7 µl of the membrane pellet and 50 µl of the first supernatant were analyzed by dodecyl sulfate gel electrophoresis. A densitometric trace of Coomassie blue stain is shown superimposed on a densitometric trace of the fluorograph from the same lane (solid line).
Methylation of Red Cell Cytoskeletal and Membrane Proteins

Identification of $^3$H-Methylated Erythrocyte Membrane Polypeptides

Cytoskeletal Polypeptides—The erythrocyte membrane is underlain by a complex of polypeptides which has been termed a cytoskeleton (cf. Refs. 4, 31–34). Table II gives a summary of the polypeptides found to be methylated in membranes of intact cells. The fact that radiolabeled methyl groups were not detected in bands 1, 2, 4.2, or 5 shows that the methyltransferase reactions are specific in intact cells. The selective methylation of the cytoskeletal polypeptides band 2.1 (ankyrin) and band 4.1 has been shown, using several different criteria, including selective extractions with lithium dioleosaliclyate and cnoate, as well as the formation of specific proteolytic products. Additionally, the formation of a soluble radiolabeled 72,000-dalton fragment derived from band 2.1 (data not shown) localizes the site of attachment of at least one methyl group to the spectrin-binding fragment.

Intrinsic Polypeptides—These polypeptides include the sialoglycoproteins, band 3, the band 4.5 region, and band 7. Two glycoproteins, detected by a periodic acid-Schiff-staining procedure and showing variable migration with respect to Coomassie blue-staining standards, are resolved in the pH 4.2 system used here and migrate at 40,000–55,000 and at 80,000–95,000 daltons. We have identified the latter species as PAS 1 because it is sensitive to trypsin digestion in intact cells. Since the radioactivity comigrating in this region is completely unaffected by trypsin digestion, we conclude that the major sialoglycoprotein (PAS 1) is not methylated. Kim et al. (11) tentatively identified a methyl-labeled component in vivo with the major sialoglycoprotein on the basis of comigration in dodecyl sulfate gels. On the basis of our evidence this represents not methylation of PAS 1 but of bands 3 and 4.1 (see above and below).

Radioactivity consistently migrates with the periodic acid-Schiff-staining polypeptide(s) at 40,000–55,000 daltons and remains associated with the membrane fraction after lithium dioleosaliclyate treatment. Neither the radioactivity nor the polypeptide(s) giving rise to the periodic acid-Schiff stain at 40,000–55,000 daltons is affected by external trypsin digestion of red blood cells, thereby excluding it from being PAS 1. We have not yet identified this glycoprotein, but the evidence presented here suggests it may be a methylated minor species of the red cell sialoglycoproteins. A small and diffuse amount of radioactivity consistently comigrates with the band 4.5 region and is not extracted by lithium dioleosaliclyate, suggesting that two or more species included in the band 4.5 region are methylated in vivo. Kim et al. (11) have also reported radioactivity migrating in this region.
Radioactivity comigrating with band 3 is apparently due to two species, both of which are intrinsic polypeptides. One is chymotrypsin-insensitive, while the other forms a 64,000-dalton fragment when intact cells are digested with chymotrypsin; hence we identify this chymotrypsin-sensitive radiolabeled species as the band 3 anion transporter (26) and membrane attachment site for the cytoskeletal shell (4). The chymotrypsin-insensitive species may be identical with the band 3' component described by Sheetz and Sawyer (32), which has also been associated with the cytoskeletal network.

Physiological Implications

The reversibility of methylation demonstrated here in polypeptides of human erythrocytes may play a key role in red cell functions in vitro. Other post-translational reversible modifications, such as phosphorylation (35), illustrate the variety of proteins modified and the different responses which may be elicited by reversible, covalent modifications. It is conceivable that the reversible methylation described here may be involved in the control of erythrocyte shape, as at least three species shown to be involved in the cytoskeletal array are methylated (bands 2.1, 3, and 4.1. The methylation of band 3 may also be relevant to its anion transporter function.

From the number of H-methyl groups incorporated/cell into bands 2.1, 3, and 4.1 (Table II), and the total number of these polypeptides/cell (36, 37), it is possible to calculate that the level of methylation of these species is 1.4, 0.2, and 0.9%, respectively. Because steady state labeling may not have been achieved and because there may have been isotopic dilution by cellular S-adenosylmethionine, these numbers represent minimal estimates of the state of methylation of these polypeptides. Nevertheless, it seems likely that only a subpopulation of these proteins may be methylated. If in fact protein methylation does modulate cell shape and deformability, then the modification of only a few molecules in the network could effect large scale cytoskeletal changes. A comparison of the species methylated in vivo with those methylated in a broken cell system (9) shows that band 3 and a comigrant, chymotrypsin-insensitive polypeptide are methylated in both systems. The cytoskeletal polypeptides which are methylated in intact cells (bands 2.1 and 4.1) are not methylated in the broken cell system. It seems reasonable to assume that a lysis of the cells disrupts the configuration of the cytoskeletal polypeptides and destroys their receptor sites. The fact that PAS 1 is methylated in a broken cell system (9), but not in intact cells, raises the possibility that nonphysiological methyltransferase reactions may occur in in vitro preparations. For example, methyl-accepting sites on the exterior membrane surface may be detected in vitro. However, with intact cells it is highly likely that only methyl acceptor sites on the cytosolic membrane surface are seen.

Acknowledgments—Special thanks are due to Dorothy Haskett and Professor Stephen Feig for help in supplying red blood cells. We are grateful to June Baumer and the Department of Biology for assistance with densitometry, and to our colleagues for their critical evaluation of this manuscript.

REFERENCES

1. Paik, W. K., and Kim, S. (1980) Protein Methylation, pp. 202-231, John Wiley & Sons, Inc., New York
2. Springer, M. S., Goy, M. F., and Adler, J. (1979) Nature 280, 279-284
3. Wang, E. A., and Koshland, D. E., Jr. (1980) Proc. Natl. Acad. Sci. U.S. A. 77, 1157-1161
4. Marchesi, V. T. (1979) Semin. Hematol. 15, 3-20
5. Kim, S. (1974) Arch. Biochem. Biophys. 181, 652-657
6. Galletti, P., Paik, W. K., and Kim, S. (1978) Biochemistry 17, 4272-4276
7. Galletti, P., Paik, W. K., and Kim, S. (1979) Eur. J. Biochem. 97, 221-227
8. O'Dea, R. F., Viveros, O. H., Acheson, A., Gorman, C., and Axelrod, J. (1978) Biochem. Pharmacol. 27, 679-684
9. Terwilliger, T. C., and Clarke, S. (1981) J. Biol. Chem. 256, 3067-3076
10. Janson, C. A., and Clarke, S. (1980) J. Biol. Chem. 255, 11640-11643
11. Kim, S., Galletti, P., and Paik, W. K. (1980) J. Biol. Chem. 255, 338-341
12. Dodge, J. T., Mitchell, C., and Haanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119-130
13. Fairbanks, G., and Avruch, J. (1972) J. Supramol. Struct. 1, 66-73
14. Bailey, J. L. (1967) Techniques in Protein Chemistry, American Elsevier Publishing Co., New York
15. Studier, F. W. (1973) J. Mol. Biol. 79, 237-248
16. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) Biochemistry 10, 2620-2627
17. Chamberlain, J. P. (1979) Anal. Biochem. 98, 132-135
18. Hajra, A. K., Seguin, E. B., and Agranoff, B. W. (1968) J. Biol. Chem. 243, 1609-1615
19. Rao, A., and Reithmeier, R. A. F. (1979) J. Biol. Chem. 254, 6144-6150
20. Liappis, N., Beeser, H., and Bantzer, P. (1979) Biot 38, 311-319
21. Winter, C. G., and Christensen, H. N. (1964) J. Biol. Chem. 239, 872-878
22. Cohn, C. K., Vesell, E. S., and Axelrod, J. (1972) Biochem. Pharmacol. 21, 803-808
23. Steck, T. L., Fairbanks, G., and Wallach, D. F. H. (1971) Biochemistry 10, 2617-2624
24. Tripplett, R. B., and Carraway, K. L. (1972) Biochemistry 11, 2897-2903
25. Marton, L. S. G., and Garvin, J. E. (1973) Biochem. Biophys. Res. Commun. 52, 1457-1462
26. Steck, T. L. (1978) J. Supramol. Struct. 8, 311-324
27. Drnckamer, L. K. (1976) J. Biol. Chem. 251, 5115-5123
28. Bennett, V., and Branton, D. (1977) J. Biol. Chem. 252, 2753-2763
29. Bennett, V. (1978) J. Biol. Chem. 253, 2299-2299
30. Steck, T. L., and Yu, J. (1973) J. Supramol. Struct. 1, 220-232
31. Bennett, V., and Stenbuck, P. J. (1979) J. Biol. Chem. 254, 2533-2541
32. Sheetz, M. P., and Sawyer, D. (1978) J. Supramol. Struct. 8, 399-412
33. Sheetz, M. P. (1979) Biochem. Biophys. Acta 557, 122-134
34. Lux, S. E. (1979) Semin. Hematol. 16, 21-51
35. Krebs, E. G., and Beavo, J. A. (1979) Annu. Rev. Biochem. 48, 923-959
36. Bennett, V., and Stenbuck, P. J. (1980) J. Biol. Chem. 255, 2540-2548
37. Steck, T. L. (1974) J. Cell Biol. 62, 1-19