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Selective Methyl Esterification of Erythrocyte Membrane Proteins by Protein Methylase II†

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ABSTRACT: Methyl esterification of erythrocyte membrane proteins has been demonstrated by incubating the isolated membrane with purified protein methylase II (*S*-adenosylmethionine:protein-carboxyl *O*-methyltransferase, EC 2.1.1.24) and *S*-adenosyl-L-[*methyl*-^{14}C]methionine. Methyl esterification of membrane-bound proteins occurred selectively to proteins corresponding to bands 3 (mol wt 97 000), 4 (mol wt 75 000), and 4.5 (mol wt 48 000) [designated according to Steck, T. L. (1974), *J. Cell Biol. 62*, 1] as identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Mild alkali treated depleted vesicles which lacked bands 1, 2, 5, and 6 had a higher methyl accepting capacity; 500 pmol of methyl groups/mg of depleted vesicle proteins vs. 200 pmol of methyl groups/mg of intact membrane proteins. Alkali-extractable membrane components were not methylated.

Protein methylase II (*S*-adenosylmethionine:protein-carboxyl *O*-methyltransferase, EC 2.1.1.24) is an enzyme which methylates (esterifies) free carboxyl groups of polypeptides (Kim and Paik, 1970; Diliberto and Axelrod, 1974; Kim, 1977). The enzyme thus neutralizes negatively charged free carboxyl groups of protein substrates by the production of protein carboxyl methyl esters. The esters are unstable and spontaneously hydrolyzed at physiological pH and temperature, yielding methanol (Kim and Paik, 1976). The enzyme has been purified from various mammalian tissues (Kim, 1973, 1974; Kim et al., 1978; Edgar and Hope, 1976). The molecular weight of the rat erythrocyte enzyme was determined to be 25 000 (Kim, 1974).

Although the enzyme is widely distributed in eukaryotes (Kim, 1977; Kim et al., 1978) and prokaryotes (Kim et al., 1977; Springer and Koshland, 1977), the identity of the natural methyl acceptor protein(s) is unknown. Furthermore, the relationship of protein methylation to enzyme function is not understood. The fact that the soluble fraction of the erythrocyte possessed a substantial amount of the enzyme despite an absence of endogenous methyl acceptor substrate (Kim et al., 1975; Kim, 1974) was particularly enigmatic. In order to investigate the endogenous methyl acceptor protein(s) in erythrocyte, we considered erythrocyte membrane proteins as possible methyl acceptors.

This paper reports the enzymatic methyl esterification of erythrocyte membrane proteins with purified protein methylase II. $NaDodSO_4$[1]–polyacrylamide gel electrophoresis of the methylated membrane enabled us to identify a few specifically methylated protein bands.

Materials and Methods

Materials. *S*-Adenosyl-L-[*methyl*-^{14}C]methionine (specific activity, 55–60 mCi/mmol) was purchased from New England

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

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Nuclear Corp. (Boston, Mass.) Ribonuclease A (five times crystallized, bovine pancrease), γ-globulin, sodium dodecyl sulfate, and 2-mercaptoethanol were obtained from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were from local sources and of the best grade available. Sprague Dawley rats (150–250 g body weight) were used for the preparation of erythrocyte membranes and protein methylase II. Protein methylase II was purified from frozen calf brain or rat erythrocyte by affinity chromatography (Kim et al., 1978).

Enzyme Assay. Methylation reactions were performed as previously reported (Kim and Paik, 1970; Kim et al., 1975), in a total incubation mixture of 0.25 mL, using the direct quantitation of methyl-^{14}C incorporation into the Cl_3AcOH-precipitable protein substrates. One unit of the enzyme was defined as 1 pmol of methyl group transferred per min at 37 °C using 5 mg of γ-globulin as the methyl acceptor protein.

Preparation of Unsealed Erythrocyte Ghosts. All the procedures were performed at 4 °C. Rat erythrocyte membranes were prepared from freshly drawn heparinized blood by the method of Steck and Kant (1974). Erythrocytes were first isolated by centrifuging the blood at 2000*g* for 10 min and washing the cells three times with 5 volumes of 0.15 M NaCl–5 mM sodium phosphate, pH 8.0. The washed erythrocytes (1 mL of packed cells) were hemolyzed by rapidly mixing with 40 mL of 5 mM sodium phosphate, pH 8.0. The ghosts were then pelleted by centrifugation at 22 000*g* for 30 min. The pellet was washed several times with the same buffer until only the faintest pink color was present. The final concentration of erythrocyte ghosts protein averaged about 5 mg/mL.

Preparation of Depleted Ghosts. Packed ghosts (2.5 mL, 13 mg of protein) were added to 35 mL of cold water with stirring. The pH was adjusted to 11.5–11.7 by the addition of 1 N NaOH. The suspension was immediately centrifuged at 22 000*g* for 30 min. The pellet, containing protein-depleted ghosts, was washed three times with the phosphate buffer and resuspended in the same buffer to a final concentration of 4 mg/mL. This membrane preparation contained between 30 and 40% of the total protein present in the intact unsealed ghosts (Steck and Yu, 1973).

Methyl Esterification of Erythrocyte Membrane Proteins. An erythrocyte membrane suspension (1 mg of protein) was incubated with 2000 units of purified protein methylase II, 40 nmol of *S*-adenosyl-L-[*methyl*-^{14}C]methionine, and 50 μL of citrate–phosphate buffer (pH 6.0) in a total volume of 0.525 mL for 2 h at 37 °C. The citrate–phosphate buffer was prepared by mixing 0.6 part of 0.25 M citric acid and 1 part of 0.5 M disodium phosphate (Kim and Paik, 1970). The ghosts were then washed several times with 5 mM sodium phosphate buffer, pH 6.8, to remove unreacted *S*-adenosylmethionine. The washed preparation was solubilized in 0.5 mL of 10 mM acetic acid containing 1 mM dithiothreitol and 2.5% $NaDodSO_4$.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Electrophoretic analyses of membrane polypeptides were performed by the method of Fairbanks et al. (1971) in 5% polyacrylamide–1% $NaDodSO_4$. Molecular weights were determined by simultaneous calibration of gels with polypeptides of known molecular weight. The electrophoresis was carried out for 4 h at room temperature. All gels were prepared in duplicate with 100–110 μg of membrane proteins. One gel was stained with Coomassie blue and then scanned at 550 nm using a Gilford 2400 spectrophotometer equipped with a Model 2410s linear-transport accessory. The other gel was sliced into 1-mm sections, and incorporated methyl-^{14}C was determined after elution of proteins from the slices. Elution was performed by soaking each 1-mm slice in 0.4 mL of 50 mM sodium acetate, pH 5.2, containing 0.25% Triton X-100 for 24 h at room temperature. The samples were then mixed with 10 mL of Formula 963 liquid scintillator (New England Nuclear). Radioactivity was determined in a Packard Tricarb Model 3375 liquid scintillation spectrometer.

TABLE I: Methyl Esterification of Rat Erythrocyte Ghosts.[a]

additions	$^{14}CH_3$ incorp (pmol/mg of protein)
intact ghosts	0
intact ghosts + calf brain protein methylase II (2000 units)	198
protein-depleted ghosts + calf brain protein methylase II (2000 units)	496

[a] One milligram of each intact and protein-depleted ghost was used as the methyl acceptor, and incubation was carried out at 37 °C for 2 h. Incubation conditions were as described in the text.

Isolation of Methylated Proteins from Erythrocyte Ghosts. The methylated and washed ghosts suspension (0.35 mL, 3.6 mg of proteins) was dissolved in 1 mL of 10 mM acetic acid–2.5% $NaDodSO_4$. The mixture was applied to a column of Sephadex G-100 (1.0 × 120 cm) equilibrated with 10 mM acetic acid–2 mM dithiothreitol–0.1% $NaDodSO_4$, with a flow rate of 10 mL/h. Fractions of 0.9 mL were collected. Radioactive fractions (fractions 31–51) were pooled and dialyzed for 2 days at room temperature against 10 mM acetic acid, lyophilized, and dissolved in 2 mL of 10 mM acetic acid. This preparation containing 16 000 cpm (139 pmol) of $^{14}CH_3$/mg of protein was used for the hydrolysis experiments.

Preparation of Ribonuclease Methyl Ester. For the methyl esterification of ribonuclease, highly purified calf brain protein methylase II was used. The incubation mixture contained 20 mg of bovine pancreas ribonuclease, 0.3 mL of citrate–phosphate buffer, pH 6, 800 units of the enzyme, and 50 nmol of *S*-adenosyl-L-[*methyl*-^{14}C]methionine in a final volume of 0.9 mL. After 1 h of incubation at 37 °C, 440 units of the enzyme and 25 nmol of *S*-adenosyl-L-[*methyl*-^{14}C]methionine were added, and the incubation was continued for an additional 2 h. The reaction was stopped by 10 volumes of cold ethanol–1 N HCl (39:1, v/v), and the protein was pelleted by centrifugation. The precipitates were washed five times with acid-ethanol and then two times with anhydrous ether and dried under vacuum. The ribonuclease methyl ester prepared by this procedure contained 18 000 cpm of $^{14}CH_3$/mg of protein.

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Results

Methylation of Erythrocyte Membrane Protein. To demonstrate the presence of membrane-bound endogenous substrate for the protein methylase II in erythrocytes, the isolated rat erythrocyte ghost was incubated with purified enzyme. An incorporation of about 200 pmol of $^{14}CH_3$/mg of membrane protein was observed, as shown in Table I. The possibility of the presence of protein methylase II in the erythrocyte membrane has also been investigated by incubating the erythrocyte ghost preparations with *S*-adenosyl-L-[*methyl*-^{14}C]methionine: No detectable protein methyl ester was formed in the absence of exogenous protein methylase II (Table I). With isolated ghosts as the methyl acceptor substrate, the enzyme showed a sharp optimum pH at 6.0 with one-half of the maximal activity at pHs 5.2 and 6.6.

Effect of Protein Substrate Concentration. Figure 1 illus-

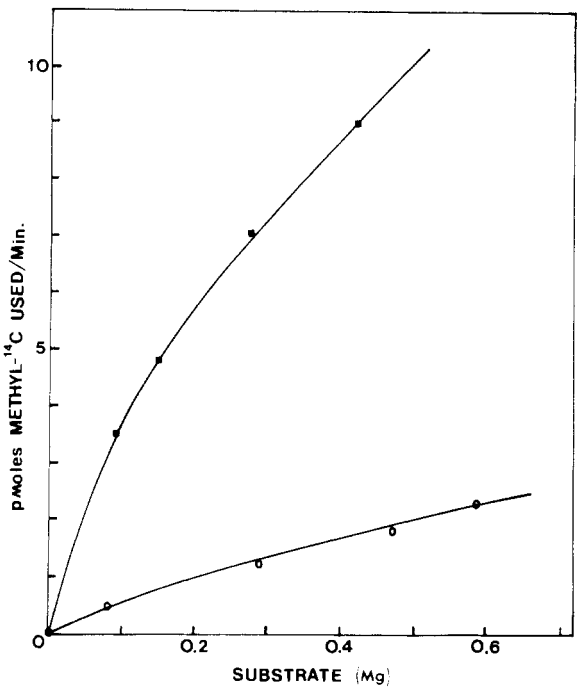

FIGURE 1: Effect of substrate concentration on the erythrocyte protein methylase II. The incubation mixture contained 75 μL of citrate–phosphate buffer (pH 6.0), 20 nmol of *S*-adenosyl-L-[*methyl*-^{14}C]methionine, 13 units of purified erythrocyte protein methylase II, and the indicated amount of substrate in a total volume of 0.25 mL: (O–O) intact erythrocyte ghosts; (■–■) protein-depleted erythrocyte ghost.

trates the effect of the concentration of membrane protein components on the protein methylase II activity. The depleted ghosts preparation which lacks bands 1, 2, 5, and 6 was a better methyl acceptor compared to the intact ghosts, 2.5 times better at the concentration of 1 mg (496 pmol vs. 198 pmol; see Table I) and about 6 times at 0.3 mg (Figure 1). This higher methyl accepting ability is most likely due to the removal of nonmethyl acceptor protein components from the membrane (Figure 3). Similar data were also obtained using a calf brain purified enzyme (data not shown), indicating that the enzyme does not exhibit any tissue specificity toward membrane components.

Characterization of the Methylated Proteins. The enzymatically methylated erythrocyte ghost was analyzed by Na-DodSO$_4$-polyacrylamide gel electrophoresis (Figure 2). Eight well-defined protein peaks were found ranging in molecular weight from 240 000 to 270 000 as reported by Steck (1974) for the human erythrocyte membrane polypeptides. The radioactivity profile of the gel indicated that only three major polypeptide peaks, corresponding to bands 3 (mol wt 97 000), 4 (mol wt 75 000), and 4.5 (mol wt 48 000), and one minor peak were methylated. The minor peak, which is present between bands 6 and 7, is most likely associated with band 7, since the depleted methylated ghost also had the minor peak (Figure 3). No radioactivity was found in the region of the two largest components, bands 1 and 2, which are known to be spectrin (Marchesi et al., 1970). Multiple determinations using different preparations of methylated ghosts gave essentially the same results.

Analysis of Depleted Methylated Vesicles. The Coomassie blue stained profile of the depleted vesicle after the Na-DodSO$_4$-polyacrylamide gel electrophoresis clearly showed the absence of bands 1, 2, 5, and 6 (Figure 3). However, the radioactivity incorporation into this depleted vesicle showed the same pattern as the intact ghost, indicating that the removal of the alkali-extractable components did not alter the qualitative methyl-accepting ability of the erythrocyte membrane, but the removal increased the extent of the methyl esterification in the remaining band (Table I). It is probably a consequence of the alkali treatment by which the methyl acceptor components in the membrane were favorably exposed for further methylation.

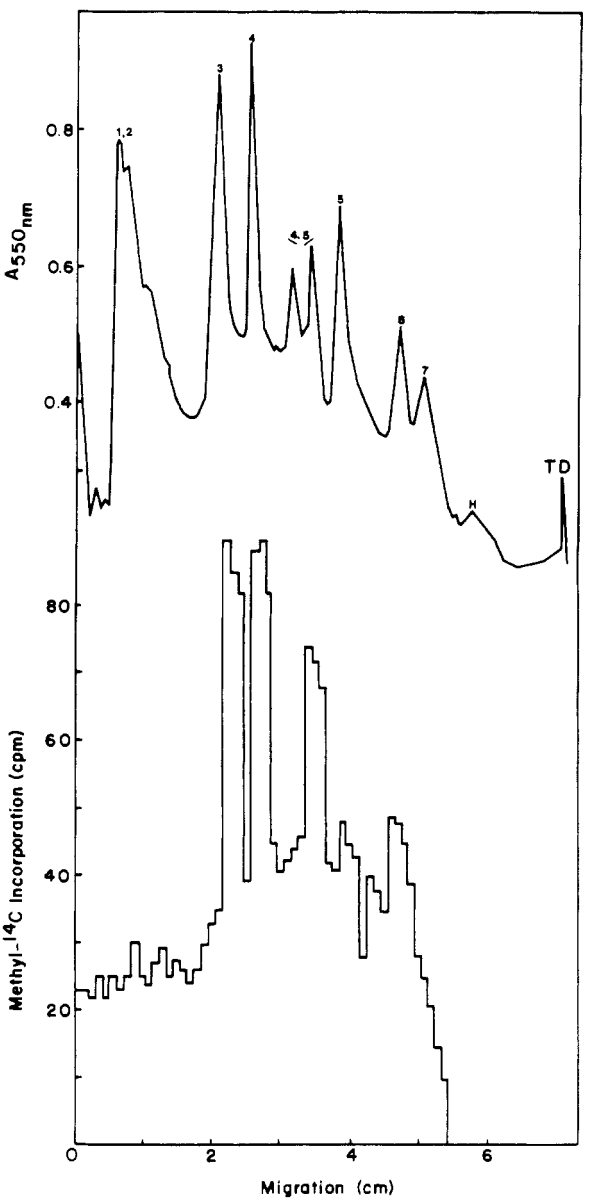

FIGURE 2: NaDodSO$_4$-polyacrylamide gel electrophoresis of erythrocyte ghosts. Upper figure, scanning at 550 nm of Coomassie blue stained gel; lower figure, radioactive profile determined on 1-mm slices. See the test for experimental details. Peak numbers are described by Steck (1974). H stands for hemoglobin.

Table II shows the specific activity of the methyl incorporation into the different protein components expressed on the basis of milligrams of methyl acceptor protein. Component 3, one of the major protein bands representing 25–30% of stained protein (Steck, 1974), corresponds to a molecular weight of 97 000. This component incorporated 87–104 pmol of methyl groups/mg of protein, while component 4 incorporated 398 pmol, about four times more than the former. The specific activity of component 4.5 cannot be evaluated accurately; however, in view of the very low degree of Coomassie blue staining together with highest radioactivity incorporated into

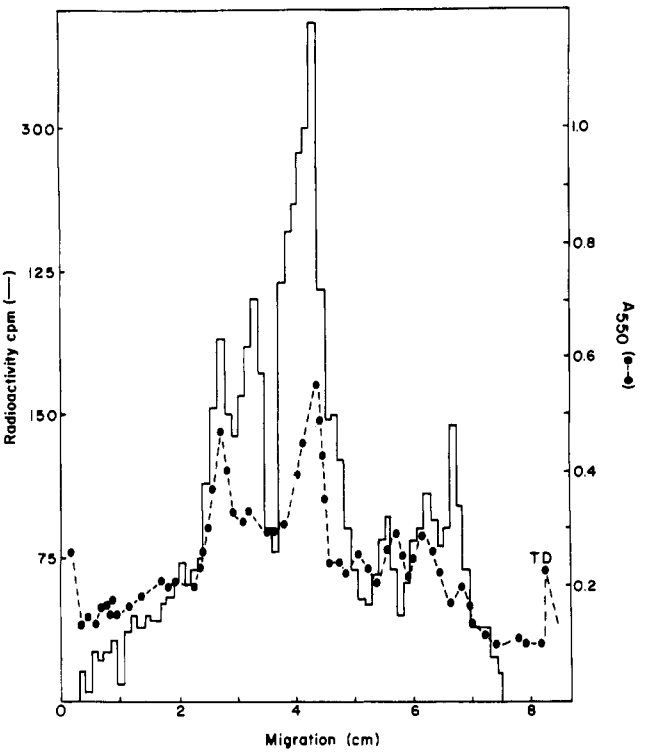

FIGURE 3: $NaDodSO_4$-polyacrylamide gel electrophoresis of protein-depleted ghosts: (●-●) scanning at 550 nm of Coomassie blue stained gel; (—) radioactive profile determined on 1-mm slices.

TABLE II: Incorporation of $^{14}CH_3$ into Erythrocyte Component Proteins.[a]

band	Coomassie blue stained (%)[c]	$^{14}CH_3$ incorp cpm	$^{14}CH_3$ incorp sp act.[b]
3	25-30	330	87-104
4	9.2	306	398
4.5		353	

[a] Membrane proteins (110 μg) with total radioactivity of 2500 cpm were charged to each gel. The values are averages of two determinations. [b] Specific activity is defined as picomole of methyl-^{14}C incorporated per milligram of the acceptor protein. [c] Data are taken from Table I in Steck (1974).

this component, it can be assumed that the specific activity will be much greater than the other two components. If band 4.5 represents about 2% of total stained protein in the membrane, the specific activity of the band would be 1400 pmol/mg.

Stability of Enzymatically Formed Methyl Esters. The protein carboxyl methyl esters formed by the action of protein methylase II are known to be unstable in aqueous alkaline solution. The half-life of the ribonuclease carboxyl methyl esters was estimated to be 25 min at pH 7.1 and 4 min at pH 8.6 (Kim and Paik, 1976), while that of membrane protein methyl ester was 2.5 min at pH 9.6 (Kleene et al., 1977). In Figure 4, the rates of the hydrolysis of erythrocyte membrane-bound protein methyl esters and ribonuclease methyl esters were compared. The relative stability of the isolated membrane-bound protein ester (upper curves) is remarkable; 90% of the esters was shown to be stable for 3 h at pH 7.0 and 75% at pH 9.0, at 37 °C. This may be partly due to the undialyzed $NaDodSO_4$ that remained in the preparation. The effect of $NaDodSO_4$ on the ribonuclease methyl ester was illustrated in Figure 5. More than 50% of the esters was protected from

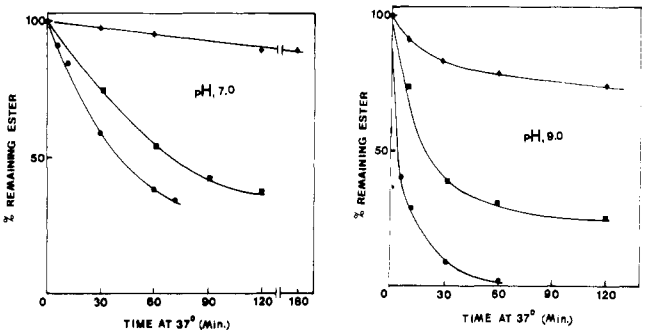

FIGURE 4: The rate of hydrolysis of ribonuclease and erythrocyte ghost carboxyl methyl esters. The hydrolyses were performed at both pH 7.0 and 9.0 at 37 °C for the indicated periods of time. Protein carboxyl methyl ester (0.5 mg) was dissolved in 0.8 mL of 0.1 M buffer and the pH was carefully checked; sodium phosphate buffer, pH 7.0, and Tris-HCl buffer, pH 9.0, were used. After the hydrolysis 50 μL of each sample was transferred to a scintillation vial, and 0.1 mL of methanol was added to each vial. The content was air-dried. After two washes with 0.2 mL of ethanol, the residues containing the nonvolatile methyl ester were dissolved in 0.2 mL of 10 mM acetic acid, 2 mM dithiothreitol, and 2.5% $NaDodSO_4$, and the radioactivity was counted with 10 mL of Formula-963 scintillation liquid: (●-●) ribonuclease carboxyl methyl ester; (■-■) erythrocyte ghost carboxyl methyl esters; (♦-♦) erythrocyte carboxyl-methylated proteins isolated by Sephadex G-100 chromatography in the presence of $NaDodSO_4$.

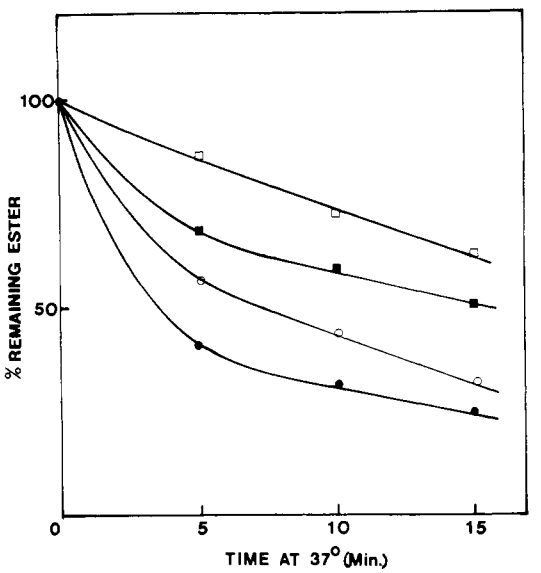

FIGURE 5: The effect of concentration of $NaDodSO_4$ on the rate of hydrolysis of ribonuclease carboxyl methyl esters. The hydrolyses were performed in 0.1 M Tris-HCl buffer, pH 9.0, for the indicated time. Estimation of the remaining ester was performed as in Figure 4: (●-●) none; (○-○) 0.05% $NaDodSO_4$; (■-■) 0.1% $NaDodSO_4$; (□-□) 0.5% $NaDodSO_4$.

the hydrolysis in the presence of 0.5% $NaDodSO_4$ at pH 9.0, indicating the $NaDodSO_4$ has an effect in stabilizing unstable protein methyl ester linkages. Although the mechanism by which the ester is protected from the hydrolysis is not known, this protective property of $NaDodSO_4$ or other related compounds may be useful in the study for the identification of the esterified dicarboxylic amino acid residues in the proteins.

Discussion

It is generally accepted that the methylation of protein plays a role in cellular control processes (Paik and Kim, 1975). In particular, the role of dicarboxylic acid methylation has been explored recently in several mammalian and bacterial systems.

Diliberto et al. (1976) proposed a possible role of the methylation of membrane protein(s) in excitation-secretion coupling of chromaffin vesicles. The modifications of the bacterial cytoplasmic membrane protein (Kort et al., 1975; Kleene et al., 1977) and the leukocyte membrane protein(s) from rabbit peritoneal exudates (O'Dea et al., 1978) were implicated as a signal for the chemotactic actions.

The results presented in this communication indicate a new class of erythrocyte membrane protein(s) which are methyl acceptors for protein methylase II. This is quite compatible with the fact that the major erythrocyte soluble protein, hemoglobin, is a poor substrate for the reaction and yet the enzyme is exclusively present in the cytosolic fraction (Kim, 1974; Kim et al., 1975). This is the first example which demonstrates the presence of methyl acceptor proteins in association with erythrocytes. Selective methyl esterification of bands 3, 4, and 4.5 observed in both intact and depleted ghosts indicates the specificity of the enzyme which only recognizes the free carboxyl groups of those proteins.

Although the significance of the specific carboxyl methylation of erythrocyte membrane proteins is presently unknown, a great potential importance of this modification is conceivable in relation to the specific function of these components. The neutralization of anionic charges of the membrane proteins by carboxyl methyl esterification and the reversible deesterification of the ester due to its labile nature might be a control mechanism in some of the functions attributed to those proteins. In this respect, it should be mentioned that band 3, one of the major components in the erythrocyte membrane, is a transmembrane glycoprotein which is involved as a site of anion transport (Ho and Guidotti, 1975; Zaki et al., 1975) or as a binding site for the glyceraldehyde-3-phosphate dehydrogenase (Yu and Steck, 1975). Recently, further evidence suggests that band 4.5, the most highly methylated band (see Figure 3), is involved in glucose transport (Kasahara and Hinkel, 1977; Goldin and Rhoden, 1978). Studies are in progress in our laboratory to investigate the role of this carboxyl methylation as a control mechanism for the aforementioned specific functions of the respective polypeptide components.

Acknowledgments

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