

Human erythrocyte membrane protein 4.2 is palmitoylated

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Protein 4.2 is a major protein of the human erythrocyte membrane. It has previously been shown to be *N*-myristoylated. After labeling of intact human erythrocytes with [³H]palmitic acid, radioactivity was found to be associated with protein 4.2 by immunoprecipitation of peripheral membrane proteins extracted at pH 11 from ghosts with anti-(4.2) sera, followed by SDS/PAGE and fluorography. The fatty acid linked to protein 4.2 was identified as palmitic acid after hydrolysis of protein and thin-layer chromatography of the fatty acid extracted in the organic phase. Protein 4.2 could be depalmitoylated with hydroxylamine, suggesting a thioester linkage. Depalmitoylated protein 4.2 showed significantly decreased binding to protein-4.2-depleted membranes, compared to native protein 4.2.

The shape and mechanical stability of the erythrocyte membrane is maintained by proteins constituting a filamentous scaffold, the membrane cytoskeleton, underlying the lipid bilayer [1, 2]. This cytoskeleton is composed mainly of spectrin tetramers held together at their junctions by short actin filaments. The interaction between spectrin and actin is strengthened by protein 4.1, which also serves as a link between the cytoskeleton and the lipid bilayer through interactions with spectrin and with glycophorin [3, 4]. The linkage of spectrin to the bilayer is also mediated by interactions of ankyrin with β spectrin and the cytoplasmic domain of band 3 [5]. In human erythrocytes, band 3 further associates with another 72000 *M_r* peripheral membrane protein, namely band 4.2. Human erythrocyte protein 4.2 is a major protein in the membrane skeletal network that represents about 5% of the total protein mass of the erythrocyte membrane (reviewed in [6]). Protein 4.2 associates with the cytoplasmic domain of the anion exchanger, band 3 [7]. It also interacts with ankyrin in solution and possibly also with protein 4.1 [7, 8]. Protein 4.2 deficiency has been related to various types of hemolytic anemias [6]. Immunoreactive forms of protein 4.2 also occur in association with the plasma membrane of non-erythroid cells [9]. Cloning of protein 4.2 cDNA has revealed that it is a member of the transglutaminase family of enzymes [10].

In chicken, rabbit, rat and human erythrocytes, several membrane proteins, including ankyrin [11], band 3 [12], a 55000 *M_r* cytoskeletal protein [13–15], protein 4.1 [16] and a subpopulation of spectrin [17], have been reported to be palmitoylated. Fatty-acid acylation of proteins confers an extra hydrophobic moiety to proteins, which promotes hydrophobic protein-membrane and protein-protein interactions [18, 19]. Protein 4.2 has recently been reported to be *N*-myristoylated [20]. We report here that protein 4.2 is also palmitoylated. We also demonstrate that depalmitoylation of

native protein 4.2 significantly decreases its binding to 4.2-depleted membrane vesicles.

MATERIALS AND METHODS

Materials

Bolton-Hunter reagent, [³H]palmitic acid and [³H]myristic acid were purchased from Du Pont/NEN. Leupeptin, aprotinin, dithiothreitol, dithioerythritol, phenylmethylsulfonyl fluoride (PhMeSO₂F), Dulbecco's modified Eagle's medium (DMEM), protein-A-bearing *Staphylococcus aureus* cells, EGTA and fatty-acid-free BSA were from Sigma Chemical Co. Q-Sepharose was obtained from Pharmacia, horse-radish-conjugated anti-rabbit IgG, Tween 20 and gelatin were from Biorad. All other reagents were of analytical grade.

Preparation and radiolabeling of cells

Blood was collected from normal, healthy human volunteers in DMEM. Cells were collected by centrifugation and washed with DMEM to remove the buffy coat. Packed erythrocytes (120 μ l) were incubated at 37°C with DMEM (8 ml) containing 20 μ Ci [³H]palmitic acid and 160.8 μ g BSA (fatty-acid-free). After 3 h, cells were pelleted, washed five times with DMEM containing 5 mg/ml BSA, twice with 155 mM NaCl, 7.5 mM sodium phosphate, 0.1 mM Na₂EDTA, pH 7.5 and lysed with lysis buffer (7.5 mM sodium phosphate, pH 7.5) containing 1 mM PhMeSO₂F, 0.1 mM leupeptin and 30 μ g/ml aprotinin. Ghosts were precipitated by centrifugation at 18 000 rpm (Sorvall SS 34 rotor) for 20 min, followed by two washes in lysis buffer.

Purification of protein 4.2

Human erythrocyte protein 4.2 was purified as described by Friedrichs et al. [9] with some modifications. Erythrocyte ghosts were incubated for 30 min at 37°C with 30 volumes 0.1 mM EGTA, pH 8.5. Membranes were pelleted at 19 000 rpm (Sorvall SS 34 rotor) for 25 min and the precipitated

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Abbreviations. DMEM, Dulbecco's modified Eagle's medium, PhMeSO₂F, phenylmethylsulfonyl fluoride.

membranes were washed once with 0.1 mM EGTA, pH 8.5. The membranes were then incubated for 30 min at 37°C with 5 volumes 1 M KCl, 1 mM dithiothreitol. Membranes were again pelleted at 19000 rpm (Sorvall SS 34 rotor) for 25 min. The precipitated membranes were collected and incubated in 5 volumes 0.1 mM EDTA, pH 11, for 45 min at 37°C. Membranes were pelleted by centrifugation at 100000 g for 60 min. The supernatant, containing protein 4.2, was used for further purification. This supernatant will be referred to as the pH 11 extract. The supernatant was dialyzed against 5 mM sodium phosphate, 0.1 mM EDTA, 0.2 mM dithioerythritol, pH 7.4 (buffer A) and loaded on a Q-Sepharose column equilibrated against the same buffer. The column was washed with 50 mM NaCl in equilibration buffer and eluted with a linear gradient of 50–400 mM NaCl in equilibration buffer. Fractions containing purified protein 4.2, as checked by SDS/PAGE, were pooled and concentrated using an Amicon Centricon-10 concentrator.

Raising of antibodies

In order to remove any minor contaminants, purified protein 4.2 was electrophoresed on 10% SDS/polyacrylamide gels; the band corresponding to protein 4.2 was excised from the gels and electroeluted. Antibodies against this purified protein were raised in adult male rabbits.

Immunoprecipitation of protein 4.2 from [³H]palmitic-acid-labeled erythrocytes

Extracts (at pH 11) were prepared from [³H]palmitic-acid-labeled erythrocyte ghosts as described above. The extract was concentrated and used for immunoprecipitation with anti-(4.2) sera as described by Firestone and Winguth [21]. 10% formalin-fixed protein-A-bearing *S. aureus* cells were washed in TETN250 immunoadsorption buffer (25 mM Tris/HCl, pH 7.5, containing 5 mM EDTA, 250 mM NaCl and 1% Triton X-100). The pH 11 extract was incubated with anti-(4.2) sera for 1 h, followed by incubation for 1 h with washed protein A-bearing *S. aureus* at 28°C with stirring. The samples were sedimented at 2500 rpm followed by three washes with 1-ml aliquots of ice-cold 5 mM sodium phosphate, pH 7.6, containing 0.5 mM EGTA, 0.5 mM dithiothreitol, 1 mM EDTA, 0.1% Triton X-100 and 1 mg/ml gelatin. The pellet was boiled for 10 min in SDS sample buffer, run on SDS/polyacrylamide gels and analyzed by fluorography as described by Bonner and Laskey [22]. Western blotting was performed as described by Towbin et al. [23].

Incubation with hydroxyamine

Incubations with hydroxyamine were carried out using various concentrations of hydroxyamine at 22°C for 30 min with the protein extract obtained at pH 11. In separate experiments, SDS/polyacrylamide gels of the [³H]palmitic-acid-labeled extracts were treated with neutral hydroxyamine (1 M) or chloroform/methanol for 6 h prior to fluorography of the gels.

Analysis of the protein-bound ³H-labeled fatty acid

Intact human erythrocytes (500 µl) were labeled with 125 µCi [³H]palmitic acid as described above. The concentrated pH 11 extract was solubilized in SDS sample buffer and electrophoresed on 10% SDS/polyacrylamide gels. Pro-

tein 4.2 was excised from the gel. The gel slices were incubated with 1 ml 1.5 M NaOH at 30°C for 3 h [24]. The pH was then adjusted to 1–2 with 6 M HCl. The hydrolyzate was extracted with chloroform/methanol and the organic phase was dried under nitrogen. The extracted lipid was analyzed by ascending chromatography on a reverse-phase C₁₈ TLC plate using acetonitrile/acetic acid (1:1, by vol.) as the mobile phase. [³H]palmitic acid and [³H]myristic acid were used as standards. The plate was scraped and the radioactivity determined in parts to determine the relative migration of radiolabeled species in the samples.

Determination of the stoichiometry of the palmitoylation of native protein 4.2 by gas-liquid chromatography

Unlabeled, purified protein 4.2 was electrophoresed on SDS/polyacrylamide gels and the band corresponding to protein 4.2 was electroeluted. Protein was estimated using the bicinchoninic-acid-assay reagent (Pierce). Protein-bound fatty acid was extracted as described above. Fatty acid methyl esters were prepared using ethereal diazomethane and analyzed by gas-liquid chromatography on a Hewlett-Packard 5890 Gas Chromatograph fitted with an integrator using a 10% diethylene glycol/succinate column (1.8 m×2 mm internal diameter) and a flame-ionization detector. Methyl pentadecanoate was used as an internal standard.

Radioiodination of protein 4.2

Protein 4.2 (native or depalmitoylated) was radiolabeled with ¹²⁵I-labeled Bolton-Hunter reagent. The Bolton-Hunter reagent was dried under a stream of nitrogen and 1 M borate, pH 8.5, was added to it. The protein to be iodinated was made 100 mM in sodium borate, pH 8.5. 125 µCi Bolton-Hunter reagent was added to 500 µg protein and the reaction was allowed to proceed on ice for 90 min. The protein was then dialyzed against 5 mM sodium phosphate, 0.5 mM EGTA, 0.5 mM dithiothreitol, 120 mM KCl, pH 8.

Preparation of protein 4.2-depleted vesicles

This was carried out as described above. The membranes remaining after extraction of band 4.2 at pH 11 were used as band-4.2-depleted vesicles.

For some experiments, vesicles were digested with 1 µg/ml chymotrypsin at a vesicle concentration of 2 mg/ml in 5 mM sodium phosphate, 0.5 mM EGTA, pH 8, at 0°C for 30 min. The reaction was terminated by adding 100 µg/ml PhMeSO₂F, and the vesicles were washed once in 5 mM sodium phosphate, 0.5 mM EGTA, pH 8.

Membrane-binding assay

This was performed as described by Korsgren and Cohen [7]. Radioiodinated protein 4.2 (0–150 µg/ml) was added to 50 µg/ml protein-4.2-depleted membrane vesicles, in a total volume of 300 µl, in a medium containing 120 mM KCl, 5 mM sodium phosphate, 0.5 mM EGTA, 0.5 mM dithiothreitol, 1 mg/ml BSA and 0.02% sodium azide. The reaction mixtures were incubated overnight (12 h) at 25°C. Two 100-µl samples were removed from each tube and centrifuged at 100000 g for 25 min. The supernatant was removed by aspiration, the protein-4.2-bound membranes were washed, pelleted again and radioactivity determined in a γ counter. At each concentration of ¹²⁵I-labeled protein 4.2, control tubes

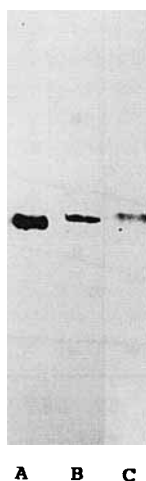


Fig. 1. Immunoprecipitation of the $[^3\text{H}]$ palmitic acid-labeled erythrocyte peripheral membrane proteins extracted at pH 11 with anti-(4.2) sera. Lane A, Western blot of purified protein 4.2 with anti-(4.2) sera. Lane B, immunoprecipitation of $[^3\text{H}]$ palmitic acid-labeled erythrocyte peripheral membrane proteins with anti-(4.2) sera, followed by SDS/PAGE and fluorography. Lane C, Western blot of erythrocyte ghosts with anti-(4.2) sera.

lacking membranes were incubated, centrifuged as above and the radioactivity determined in order to account for protein 4.2 adhering to the walls of the tubes. For some experiments, chymotrypsin-treated vesicles were used and the membrane-binding assay was carried out as described above.

Competition by unlabeled band 4.2 for binding of ^{125}I -labeled band 4.2 to membranes stripped at pH 11

^{125}I -labeled band 4.2 (36 $\mu\text{g}/\text{ml}$) was incubated with 50 $\mu\text{g}/\text{ml}$ proteins stripped from membranes by exposure to pH 11 and the indicated concentrations of unlabeled band 4.2. Membrane-binding assays were performed as described above.

RESULTS

Identification of the $[^3\text{H}]72000\text{-}M_r$ protein obtained after labeling of human erythrocytes with $[^3\text{H}]$ palmitic acid

In order to identify the 72000- M_r protein that is labeled on incubation of human erythrocytes with $[^3\text{H}]$ palmitic acid, $[^3\text{H}]$ palmitic-acid-labeled erythrocyte ghosts were subjected to extraction of peripheral membrane proteins as described above. After concentration, the pH 11 extract was subjected to immunochemical analysis using anti-(4.2) sera. Fig. 1 shows that the antisera was specific for protein 4.2. The antigen-antibody complex obtained after incubation of the labeled pH 11 extract with anti-(4.2) sera, was precipitated using protein-A-bearing *S. aureus* cells. The pelleted proteins were analyzed by SDS/PAGE, followed by fluorography. A 72000- M_r band was obtained after fluorography, confirming that the polypeptide labeled in that region is indeed protein 4.2.

Identification of the fatty acid associated with protein 4.2

In order to recover the protein-4.2-linked fatty acid from the protein, the radiolabeled pH 11 extract was electrophore-

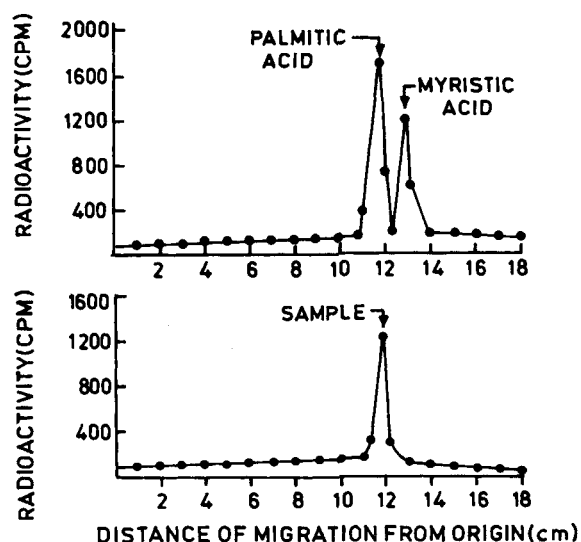


Fig. 2. Identification of fatty acids after hydrolysis of human erythrocyte protein 4.2 recovered from $[^3\text{H}]$ palmitic acid-labeled erythrocytes. The organic extract of hydrolysed protein 4.2 was dried and run on reverse-phase C_{18} TLC plates with $[^3\text{H}]$ myristic acid $[^3\text{H}]$ palmitic acid as standards. After ascending chromatography, samples 1 cm in height and 2 cm in width were scraped from the plate and radioactivity determined.

resed on SDS/polyacrylamide gels and protein 4.2 was excised from the gels. The gel slices were subjected to alkaline hydrolysis, extraction of the fatty acid in the organic phase and reverse phase TLC. The labeled species comigrated with $[^3\text{H}]$ palmitic acid, demonstrating that the fatty acid incorporated into band 4.2 is palmitic acid (Fig. 2). Association of palmitic acid with native, unlabeled protein 4.2 was further confirmed by gas-liquid chromatography of the fatty acid liberated from unlabeled protein 4.2. The molar ratio of protein 4.2/palmitic acid was found to be 1:2. However, this estimate should be treated with caution due to background contamination probably arising from the detergent associated with the electroeluted protein 4.2 from which the fatty acid was extracted.

Sensitivity of the protein-4.2-linked fatty acid to cleavage by hydroxylamine

In order to depalmitoylate protein 4.2, the pH 11 extract was treated with different concentrations of hydroxylamine for 30 min at 22°C. This treatment did not lead to any alterations in proteins as evidenced by Coomassie blue staining of the treated and untreated pH 11 extracts. However, hydroxylamine at a concentration of 1 M caused depalmitoylation of protein 4.2 as assessed by SDS/PAGE and fluorography of treated and untreated $[^3\text{H}]$ palmitic-acid-labeled pH 11 extracts (Fig. 3). Gels of $[^3\text{H}]$ palmitic-acid-labeled peripheral protein extracts obtained at pH 11 were also treated with chloroform/methanol or neutral hydroxylamine and subjected to fluorography. The band-4.2-associated label was insensitive to chloroform/methanol, but sensitive to neutral hydroxylamine (data not shown). This suggested that the fatty acid was linked by a thioester bond to band 4.2.

Binding of $[^{125}\text{I}]$ band 4.2 to band-4.2-depleted membranes

Binding was studied using both native and depalmitoylated protein 4.2. In the case of depalmitoylated band 4.2, the

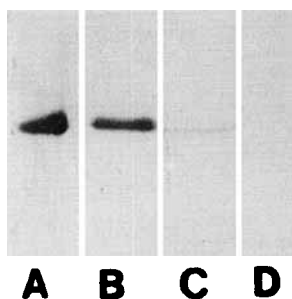


Fig. 3. Treatment of [^3H]palmitic acid-labeled erythrocyte membrane proteins extracted at pH 11 with hydroxylamine at 22°C for 30 min. Peripheral membrane proteins obtained after labeling of cells with [^3H]palmitic acid were treated with hydroxylamine at 22°C for 30 min, followed by SDS/PAGE and fluorography. Lane (A) control; lane (B) 0.25 M hydroxylamine; lane (C) 0.5 M hydroxylamine; lane (D) 1 M hydroxylamine.

pH 11 peripheral protein extract was immediately dialyzed against buffer A at 4°C after treatment with 1 M hydroxylamine as described above. The dialyzate was used for purification of depalmitoylated protein 4.2 by chromatography on Q-Sepharose as described above. Both native and depalmitoylated band 4.2 were labeled with Bolton-Hunter reagent as described in Materials and Methods. Both native and depalmitoylated protein 4.2 bound to the membranes in a saturable manner (Fig. 4 A). The possibility of studying non-specific binding in these cases was ruled out by the fact that the binding of [^{125}I]band 4.2 to chymotrypsin-treated vesicles (representing non-specific binding) was only about 15% of the binding of native band 4.2 to the membranes (Fig. 4A). Moreover, increasing concentrations of unlabeled band 4.2 competed with [^{125}I]band 4.2 for binding to pH-11-stripped membranes (Fig. 5).

The binding of depalmitoylated band 4.2 was significantly lower than that of native band 4.2. Scatchard analysis of the binding data shown in Fig. 4A gave a concave plot for native band 4.2 (Fig. 4 B), as described in [7], demonstrating high-affinity and low-affinity binding of band 4.2 to the membranes. In the case of depalmitoylated protein 4.2, the high-affinity-binding component was not observed. Extrapolation of the curve on the x axis, gave a binding capacity of 280 mg band 4.2/g vesicle protein in our experiments with native band 4.2. However, this was reduced to 108 mg band 4.2/g vesicle protein in the case of depalmitoylated band 4.2.

DISCUSSION

Protein 4.2 is one of the major proteins of the human erythrocyte membrane, representing 5% of the protein. Protein 4.2 is essential for the normal functioning of the erythrocyte membrane, since defects and deficiencies in band 4.2 result in fragile and abnormally shaped erythrocytes, leading to hemolytic anemia [25–27]. Protein 4.2 associates with the cytoplasmic domain of band 3 [7] and also with ankyrin [8]. Band 3 appears to represent the major high-affinity binding site for protein 4.2 on the erythrocyte membrane [7].

Although protein 4.2 is a peripheral membrane protein, it is resistant to extraction from the membrane, unless harsh conditions such as high pH or mildly chaotropic agents are used [28]. Myristoylation of protein 4.2 probably contributes towards this strong interaction with the membrane [20]. We now demonstrate that, in addition to being myristoylated, band 4.2 is also palmitoylated.

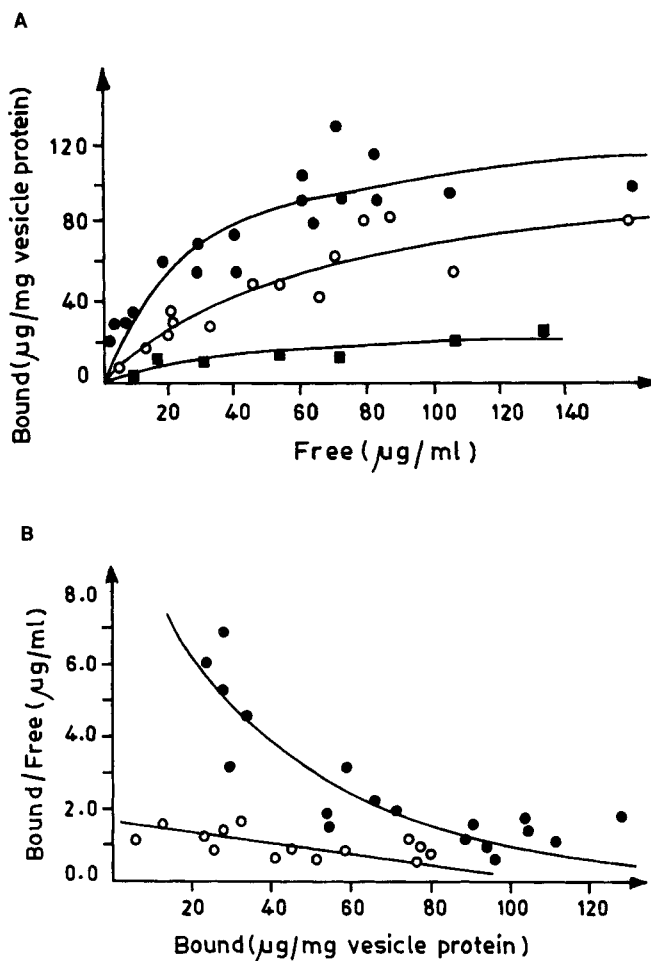


Fig. 4. Analysis of the binding of band 4.2 to band 4.2-depleted vesicles. (A) Binding of [^{125}I]labeled band 4.2 to pH 11-stripped (i.e. band 4.2-depleted) vesicles. The binding was as described in Materials and Methods. (●) Control band 4.2; (○) depalmitoylated band 4.2; (■) binding to pH-11-stripped vesicles digested with 1 μg/ml chymotrypsin. (B) Scatchard plots of the binding data shown in (A). Data were analyzed using the software ENZFITTER (Elsevier Bio-soft).

Palmitoylation is found in diverse membrane proteins including viral glycoproteins [29, 30], the β_2 adrenergic receptor [31], the acetylcholine receptor [32], rhodopsin [33, 34], ras proteins [35], the transferrin receptor [36] and the protein tyrosine kinase p56^{lck} [37]. Palmitoylation not only serves to anchor proteins to membranes [38], but also plays such important roles as regulation of protein-protein interactions [31], regulation of receptor-mediated endocytosis of the transferrin receptor [39] and phosphorylation of transglutaminase [40]. Erythrocyte membrane proteins which are known to be palmitoylated include p55, a peripheral membrane protein of human erythrocytes [13–15], band 3 [12], chicken and rabbit erythrocyte ankyrin [11], rat erythrocyte band 4.1 [16] and the Rh antigen [41, 42].

In the present investigation, immunoprecipitation of the [^3H]palmitic acid-labeled 72000- M_r protein of the human erythrocyte membrane with anti-(4.2) sera has confirmed that protein 4.2 is acylated. Analysis of the 4.2-linked fatty acid has shown that it is palmitoylated. Since mature human erythrocytes lack a protein synthesising machinery, the metabolic labeling of protein 4.2 with [^3H]palmitic acid must be,

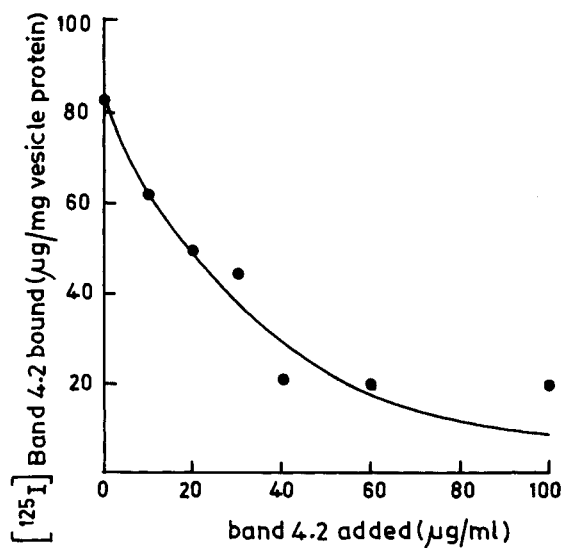


Fig. 5. Competition by unlabeled band 4.2 for binding of [^{125}I]band 4.2 to membranes. Binding was measured as described in Materials and Methods.

as expected, a post-translational modification. The sensitivity of the protein–palmitic-acid linkage to cleavage by hydroxylamine suggests that the palmitic acid is present in the usual thioester linkage.

Depalmitoylation with hydroxylamine has been used to study the functional properties of rhodopsin [43] and the influenza virus spike glycoprotein [44]. The questions of conformational changes which are associated with the technique of site-directed mutagenesis do not arise in such experiments. We have utilized a similar approach to depalmitoylate protein 4.2. The association of depalmitoylated protein 4.2 with protein-4.2-depleted membranes is significantly decreased compared to native protein 4.2. It has previously been reported that there is a high-affinity and a low-affinity binding component for protein 4.2 binding to membranes [7]. Scatchard analysis of the binding data for control and depalmitoylated protein 4.2 suggests that the high-affinity binding component is affected by palmitoylation. Whereas control protein 4.2 showed a binding capacity of 280 mg/g vesicle protein in our experiments, depalmitoylated protein 4.2 gave a value of 108 mg/g vesicle protein. Korsgren and Cohen suggested that the high-affinity binding component represents band 3 on the membrane, since this component is affected when band 3 on the membrane is digested with chymotrypsin. Our findings raise the possibility that the concave Scatchard plot may also be due to the heterogeneity in native protein 4.2 with respect to its extent of palmitoylation. Covalent attachment of palmitic acid to proteins may modulate protein-protein interactions. Assuming that the high-affinity binding site is band 3, it is possible that palmitoylation of protein 4.2 favours its interaction with band 3 on the membrane. Binding studies with band 3 in solution will be needed to resolve this issue.

Band 4.2 has extensive similarity with the Ca^{2+} -dependent cross-linking proteins, the transglutaminases [45], and the two protein classes probably arose from a common ancestral gene [10]. Interestingly, keratinocyte transglutaminase has also been reported to be both myristoylated and palmitoylated [46]. It has further been demonstrated that transglutaminase mutants in which the cysteine cluster responsible for palmitic acid thioesterification, is deleted, are cytosolic

[40]. The protein tyrosine kinase p56^{lc} of T cells is also both myristoylated and palmitoylated [37].

In conclusion, the present data suggest that palmitoylation of protein 4.2 modulates its binding to the erythrocyte membrane. A tightly membrane-associated palmitoylated form of spectrin probably represents a phosphoform of spectrin [17]. Further investigations into the relationship between palmitoylation and phosphorylation of protein 4.2 may provide insight into the regulatory significance of these post-translational modifications.

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