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The topology of the major band 4.5 protein component of the human erythrocyte membrane: characterization of reactive cysteine residues

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A preparation of band 4.5 protein of the red cell membrane, containing largely the sugar transporter, was labelled with the sulfhydryl reagent *N*-ethyl[^{14}C]maleimide. In preparations denatured with sodium dodecyl sulfate (SDS), all five sulfhydryl groups present in the peptide, M_r 45000 to 60000, react with the alkylating agent within 20 min at 37°C. If the peptide is reconstituted in lipid vesicles and cleaved with trypsin before extraction and denaturation with SDS, three sulfhydryl groups are found in a 30 kDa fragment and two in a 19 kDa fragment. In 'native' reconstituted protein only three groups react, even after two hours of exposure, two in the 30 kDa fragment and one in the 19 kDa fragment. Thus, one sulfhydryl group is cryptic, inaccessible to *N*-ethylmaleimide in each fragment. In intact cells, the single reactive group of the 19 kDa fragment can be protected against reaction with *N*-ethylmaleimide by the impermeant sulfhydryl reagent, *p*-chloromercuribenzene sulfonate (PCMBS). It is, therefore, considered to be exposed on the outer face of the membrane. The two reactive groups of the 30 kDa fragment are not protected by PCMBS and are, therefore, not considered to be exposed to the outside medium. Cytochalasin B, a competitive inhibitor of sugar transport affords temporary protection of the exofacial group of the 19 kDa against reaction with *N*-ethylmaleimide, and affords longer term protection of one of the reactive groups of the 30 kDa fragment. These findings allow conclusions about the topology of the sugar transport protein in the bilayer. Both proteolytic fragments must cross the bilayer. One of three reactive sulfhydryl groups is exofacial and two may be cytoplasmic. The two cryptic groups may be located within the bilayer.

Introduction

The facilitated diffusion of hexoses across the plasma membrane of the human erythrocyte has been studied extensively [1,2]. Despite initial questions concerning the identity of the transport protein [3–6], recent studies [7–11] provide strong evidence that a component of band 4.5, a broad band in sodium dodecyl sulfate (SDS)-acrylamide gels of approx. 50 to 60 kDa, is responsible for hexose transport and contains the binding site for cytochalasin B, a potent inhibitor of sugar transport [2,3]. Taking advantage of a photoaffinity labelling method for covalent interaction of cytochalasin B with its binding site, and the relative ease with which a sugar-transporting component

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Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PCMBS, *p*-chloromercuribenzene sulfonate; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid.

of band 4.5 can be isolated and reconstituted into liposomes composed of erythrocyte membrane lipids, we and others have employed limited proteolysis of the photolabelled transporter in intact red blood cells, erythrocyte membranes, and reconstituted liposomes in attempts to deduce the transmembrane topology of the transport protein [12–15]. With only minor differences, it was reported that in intact cells band 4.5 is resistant to the action of trypsin, but that cleavage at the membrane's cytoplasmic face produces two membrane-bound fragments, one with a molecular mass of approx. 30 kDa that contains the carbohydrate moiety of the glycoprotein, and one non-glycosylated component of 19 kDa that retains covalently incorporated cytochalasin B.

This approach combining specific labelling with proteolytic dissection can potentially provide useful information concerning the location of other sites on the transporter molecule. The cysteine residues of preparations of 'purified' sugar transporter are particularly suitable sites for such an investigation. Firstly, specific labelling of sulfhydryl groups with chemical reagents is well documented [16]. Secondly, the transporter contains a total of only five cysteine residues [17], a manageable number compared to other more numerous amino acids. Most importantly, previous studies have shown that a number of sulfhydryl-modifying reagents can inhibit hexose transport in intact erythrocytes [18–23], and that cytochalasin B can protect the transporter against this inactivation. Several of these studies [19,22,23] have provided evidence that modification of a sulfhydryl group (or groups) located on the exterior face of the cell inhibits sugar transport.

In the present study, we have attempted to characterize the cysteine residues in a band 4.5 preparation by enumerating the sulfhydryl groups that react with the transport inhibitor *N*-ethylmaleimide after denaturation with sodium dodecyl sulfate or after its reconstitution in the native state in lipid vesicles. The former provides information concerning the total number of sulfhydryl groups and the latter, the groups that are *N*-ethylmaleimide-reactive in the 'native' protein in a membrane environment. The distribution of total and reactive residues on the two tryptic fragments of the transport protein was determined, as well as those residues protected against *N*-ethylmaleimide interaction by the impermeant reagent *p*-chloromercuribenzenesulfonic acid (PCMBS), or by the sugar transport inhibitor, cytochalasin B. The results allow additional conclusions concerning the topology of the transport protein.

Materials and Methods

Recently outdated human blood was used in these studies. *N*-Ethyl[^{14}C]maleimide was obtained from New England Nuclear (42.9 mCi/mmol) or Amersham (7 mCi/mmol). Trypsin (TPCK treated), cytochalasin B, cytochalasin E, *p*-chloromercuribenzenesulfonic acid (PCMBS), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), DEAE-Sephacel, molecular weight standards (SDS-7), and sodium dodecyl sulfate (SDS) were purchased from Sigma. Electrophoresis reagents were from Bio-Rad. All other chemicals were reagent grade.

Preparation of a band 4.5 fraction containing the sugar transport peptide. Human erythrocytes were collected from whole blood by centrifugation and washed three times with five volumes of phosphate-buffered saline (5 mM sodium phosphate, pH 8, 150 mM NaCl, taking care to remove the buffy coat. The washed cells were lysed in 15 volumes of 5 mM sodium phosphate (pH 8), and washed in that buffer until they appeared white. Peripheral proteins were removed by washing the ghosts twice with 20 volumes of 0.01 M NaOH/0.1 mM EDTA [24]. Band 4.5 peptides were isolated from the alkali-treated membranes by the method of Baldwin et al. [17] slightly modified [12]. Briefly, the membranes (2 mg protein/ml) were extracted with 1% *n*-octyl glucoside in 50 mM Tris-HCl (pH 7.4), 1% β-mercaptoethanol and centrifuged at $100\,000 \times g$ for 60 min. The supernatant solution was applied to a column of DEAE-Sephacel equilibrated with 1% octyl glucoside in 50 mM Tris-HCl (pH 7.4), and eluted with that buffer. The column eluate, which contained band 4.5 and membrane lipids that were not retarded by the anion exchange column, was dialyzed against three changes of 200 volumes of phosphate-buffered saline containing 0.1 mM EDTA to remove the detergent and thereby to allow the formation of vesicles. The reconstituted vesicles, containing the sugar trans-

porter, were concentrated by centrifugation at $100\,000 \times g$ for 1 h, and resuspended in phosphate-buffered saline at a concentration of 1.5–2 mg protein/ml.

Labelling with N-ethylmaleimide. *N*-Ethyl-[^{14}C]maleimide was transferred from a stock solution in pentane to an aqueous solution by vortexing an aliquot of the stock with phosphate-buffered saline and removing the upper pentane layer with a gentle stream of nitrogen. Radiolabelled *N*-ethylmaleimide was used without dilution with unlabelled reagent. Solutions of *N*-ethyl[^{14}C]maleimide and suspensions of reconstituted band 4.5 preparation were mixed to yield a final concentration of 15 mM *N*-ethyl[^{14}C]maleimide and 0.8 mg protein/ml band 4.5 preparation. In some experiments, 2% SDS was added to solubilize and denature the protein. Samples were incubated at 37°C for the reaction time indicated in each figure, and the reaction was terminated by the addition 45 mM β-mercaptoethanol and cooling the samples to 0°C.

Tryptic cleavage. In experiments designed to determine the distribution of cysteine residues between the 30 kDa and 19 kDa fragments produced by trypsin treatment of the band 4.5 preparation, *N*-ethyl[^{14}C]maleimide-labelled reconstituted preparation (0.5 mg/ml) was incubated with 5 μg/ml trypsin for 90 min at 25°C. Proteolysis was stopped by the addition of 50 μg/ml of the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF) and 1% SDS. For the study of SDS-denatured transporter, the peptide in vesicles was cleaved with trypsin prior to denaturation and reaction with *N*-ethyl[^{14}C]maleimide.

Identification of exofacial sulfhydryl groups. Cysteine residues exposed at the exterior of intact erythrocytes were identified on the basis of protection against reaction with *N*-ethylmaleimide by a relatively impermeant sulfhydryl specific agent, *p*-chloromercuribenzene sulfonate (PCMBS). This agent inhibits sugar transport by binding to exofacial sulfhydryl groups [19]. It permeates the membrane very slowly, but this process is inhibitable by the anion transport inhibitor 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) [25], without effect on sugar transport [26]. Washed erythrocytes (25% hematocrit in phosphate-buffered saline) were treated with 12 μM DIDS for 1 h at 37°C. The cells (10% hematocrit) were then incubated with 0.1 mM PCMBS for 1 h at 0°C to allow interaction with superficial sulfhydryl groups.

Erythrocytes were washed twice with 10 volumes of the saline solution to remove unbound reagent. Because of the exceptionally high affinity of the mercurial-mercaptide bond, virtually no PCMBS is released from the membrane sulfhydryl groups by saline washing. Cells resuspended to 10% hematocrit in saline solution were exposed to 15 mM non-radioactive *N*-ethylmaleimide, and incubated at 37°C for 1 h, to allow reaction with 'unprotected' cysteine residues, i.e. those that are located inside of the membrane, inaccessible to PCMBS. The cells were then washed twice with 10 volumes of phosphate-buffered saline, once with saline containing 1% β-mercaptoethanol (a sulfhydryl containing reagent) to scavenge unreacted *N*-ethylmaleimide and to remove PCMBS from the protected sites, and twice with saline. Membranes were prepared from these and from control cells that were treated identically except that the incubation with PCMBS was omitted. The band 4.5 preparation was isolated from these membranes, reacted with *N*-ethyl[^{14}C]maleimide, and cleaved with trypsin as described above. By the above procedure only the exofacial cysteine residues, protectable by PCMBS should be labelled.

Electrophoretic analysis of N-ethylmaleimide-labelled peptide. The incorporation of *N*-ethyl[^{14}C]maleimide into band 4.5 peptide and its tryptic fragments was monitored electrophoretically. Samples were solubilized in a medium containing 1% SDS, 5% glycerol and 5% β-mercaptoethanol, and analyzed by SDS-slab gel electrophoresis as described by Laemmli [27], with a stacking gel of 4.5% polyacrylamide and a separating gel of 12.5% polyacrylamide. After fixing and staining the gels with Coomassie blue, 3 mm slices were cut and digested with 0.5 ml 30% H_2O_2 for 2 h at 80°C. The samples were cooled to room temperature, and 15 ml Aquasol 2 (New England Nuclear) was added. Radioactivity of *N*-ethyl[^{14}C]maleimide in each gel slice was determined by liquid scintillation counting, with a counting efficiency of 85% under these conditions. Molecular weights were assigned based on standards using a series of peptides of known size.

In some instances, the binding of *N*-ethylmaleimide was calculated in moles of reagent per mole of peptide. These calculations were based on the following: (1) moles of bound *N*-ethylmaleimide were determined on the basis of total counts in gel slices of particular peptide bands, after correction for counting efficiency, using the known specific activity of the *N*-ethyl[^{14}C]maleimide; (2) the recovery of *N*-ethylmaleimide in the peptide fractions was determined from the total counts in the gel slices compared to the count applied to each gel; (3) the amount of peptide applied to each gel was based on determinations by the procedure of Lowry et al. [28] using bovine serum albumin as a reference standard; (4) it was assumed that all of the applied peptide was resolved in the gel and that the *N*-ethyl[^{14}C]maleimide was bound only to the sugar transport component; (5) it was assumed that the peptide backbone of the transport peptide is 46 kDa in size, based on its mobility in SDS-gel electrophoresis after removal of most of its carbohydrate with endoglycosidase F [15,29].

The calculated values must be considered as estimates because they are subject to certain errors including: precise assignment of counts to particular peptide bands is difficult because of overlaps; peptides other than the sugar transporter may contribute up to 30% of the band 4.5 preparation [12,15,17]; and the assignment of molecular weights based on SDS-acrylamide electrophoresis. On the other hand, the band 4.5 peptide preparation contains only five cysteine residues [17] and its tryptic fragments, a smaller number. The calculated data appear to be sufficiently accurate to determine the appropriate whole number integers, representing the number of *N*-ethylmaleimide-binding sites per mole of intact peptide and its tryptic fragments.

Other methods. Spectrophotometric titration of sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoic acid) [30] served as a second means of determining the total number of cysteine residues in band 4.5. Samples (0.3 mg protein/ml) were solubilized with 2% SDS prior to reaction.

Results

As already noted, each mole of band 4.5 preparation is reported to contain five cysteine residues [17]. This value was confirmed by spectrophotometric titration of sulfhydryl groups in the band 4.5 preparation with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (4.70 ± 0.25, $n = 4$). Although, as mentioned above, the band 4.5 preparation does not consist solely of the glucose transporter, Baldwin et al. [17], noting that N-terminal and C-terminal analyses of band 4.5 detected only a single acid residue at each terminus, and that the preparation appears to bind 0.8 moles of cytochalasin B per mole of peptide, have concluded that polypeptides other than the glucose transporter must comprise less than 20% of the band 4.5 preparation. Moreover, we [15] and others [29] have previously reported that removal of the carbohydrate moiety from the protein converted

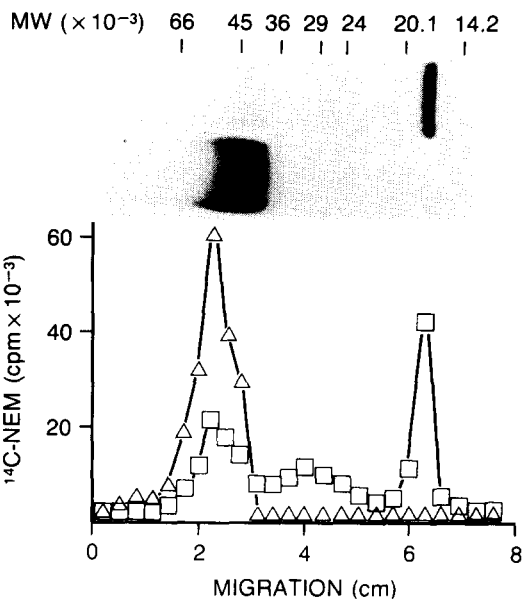

Fig. 1. Labelling of the cysteine residues of SDS-solibulized (denatured) band 4.5 preparation with *N*-ethyl[^{14}C]maleimide ([^{14}C]-NEM). Intact (△) and trypsin-cleaved (□) peptide was solubilized with 2% SDS and labelled with 15 mM *N*-ethyl[^{14}C]maleimide as described in Materials and Methods, and electrophoresed according to the method of Laemmli [27] with a stacking gel of 4.5% acrylamide and a separating gel of 12.5% acrylamide. The amount of radioactivity in 3 mm slices of the gels is represented. A photograph of Coomassie blue stained electrophoretograms of the intact (lower lane) and trypsin-cleaved (upper lane) protein are shown above. The numbers at the top represent the locations of molecular weight standards.

virtually all of the material seen in the diffuse electrophoretic pattern of band 4.5 to a sharply defined band ($M_r = 46\,000$). Thus, determination of the sulfhydryl content of band 4.5 provides a reasonable estimate of the number of cysteine residues present in the hexose transporter, the major component of the band 4.5 preparation.

All five cysteine residues appear to react with *N*-ethylmaleimide in SDS-denatured preparations. A typical electrophoretic profile of radioactivity of the *N*-ethylmaleimide-labelled protein is illustrated in Fig. 1 (intact peptide). A broad band of radioactivity extending from 45 kDa to 70 kDa was observed, coinciding with the equally broad Coomassie blue staining profile of band 4.5. Assuming a molecular mass of 46 kDa for the carbohydrate-free polypeptide of the hexose transporter [15,29], approx. five moles of *N*-ethyl[^{14}C]maleimide were incorporated per mole of band 4.5 (5.22 ± 0.11, $n = 4$). The exposure of the transporter to *N*-ethylmaleimide was for 20 min, sufficient time for maximal labelling (see below).

As previously reported [12–14], proteolysis of the reconstituted band 4.5 preparation with trypsin yields only two major products: a fragment of approx. 30 kDa that contains the transporter's carbohydrate moiety, and a non-glycosylated fragment of 19 kDa. The upper gel track of Fig. 1 illustrates the cleavage pattern. Some uncleaved peptide is found in the usual location of band 4.5. The broad, lightly stained product located at about 30 kDa is the glycosylated fragment, and the sharp, heavily stained band at 19 kDa is the carbohydrate-free peptide.

Tryptic proteolysis of the reconstituted transporter resulted in the redistribution of the *N*-ethyl[^{14}C]maleimide label (Fig. 1, trypsin-cleaved). The labelling in this case was done after proteolysis, using SDS-denatured peptide so that all five cysteine residues were modified. The amount of label in the band 4.5 region (the parent peptide) was substantially diminished, but a broadly distributed region of radioactivity corresponding to the 30 kDa fragment and a much sharper band that co-migrated with the 19 kDa fragment appeared.

The distribution of *N*-ethyl[^{14}C]maleimide in the various regions of the gels was quantified by estimating areas under the labelled peaks, as described in Materials and Methods. Comparison of intact and trypsin-cleaved preparations indicated that proteolysis was incomplete even after prolonged exposure to trypsin ($69 \pm 3\%$, $n = 15$). More than 95% of the *N*-ethyl[^{14}C]maleimide was recovered in the 50, 30 and 19 kDa fragments, indicating that any small peptides that might be released from the protein [15] or peptides derived from components of band 4.5 other than the hexose transporter do not contain an appreciable fraction of the total sulfhydryl content of the band 4.5 preparation. Based on molecular mass values of 30 and 19 kDa, the proteolytic fragments were calculated to bind 2.83 ± 0.15 and 2.42 ± 0.08 moles of *N*-ethyl[^{14}C]maleimide, respectively ($n = 3$).

Reaction of *N*-ethylmaleimide with the cysteine residues in SDS-denatured peptide proceeded rapidly. All five sulfhydryl groups reacted with the alkylating agent within 20 min (Fig. 2). In contrast, the cysteine residues of the native, reconstituted transporter reacted at a much slower rate. After 2 h, only about three moles of *N*-ethyl[^{14}C]maleimide were incorporated per mole

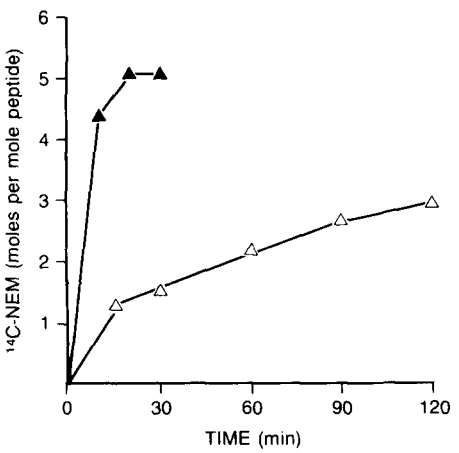

Fig. 2. Time-course of incorporation of *N*-ethyl[^{14}C]maleimide ([^{14}C]-NEM) into denatured and 'native' reconstituted band 4.5 preparations. Membrane reconstituted (△) and SDS-solubilized (▲) preparations of peptide were incubated with 15 mM *N*-ethyl[^{14}C]maleimide for the times indicated. The reaction was terminated by addition of 45 mM β-mercaptoethanol. The radioactivity incorporated into band 4.5 was determined electrophoretically as in Fig. 1, and the data were calculated on the basis of moles of *N*-ethylmaleimide per mole of peptide as described in Materials and Methods.

of peptide (2.88 ± 0.28, $n = 3$). The labelled 'native' peptide, after trypsin treatment, displayed a *N*-ethyl[^{14}C]maleimide profile similar to that found with denatured peptide (Fig. 1) except that the amount of label was diminished. The 30 and 19 kDa fragments contained 1.85 ± 0.21 and 1.03 ± 0.18 moles of *N*-ethylmaleimide per mole of peptide, respectively ($n = 3$). For comparative purposes all of the data on *N*-ethylmaleimide content of intact band 4.5 and its proteolytic fragments are compiled in Table I.

N-Ethylmaleimide rapidly permeates the red cell membrane [20], so it might interact with sulfhydryl groups located on either the outside or cytoplasmic faces of the membrane. Impermeant sulfhydryl reagents, including analogs of *N*-ethylmaleimide [22,23] and the mercurial PCMBS [19], can inhibit sugar transport presumably by binding to an exofacial group. The topology of the *N*-ethylmaleimide binding sites in the transporter was investigated by using PCMBS to 'protect' exofacial sites against reaction with *N*-ethylmaleimide. The procedure used (described in Materials and Methods) results in suppression of labelling of 'unprotected' sites, so that the 'protected', exofacial sites, should be labelled to a greater degree. The labelling profiles of trypsin-cleaved band 4.5 derived from PCMBS-protected and control cells are shown in Fig. 3. The general patterns are

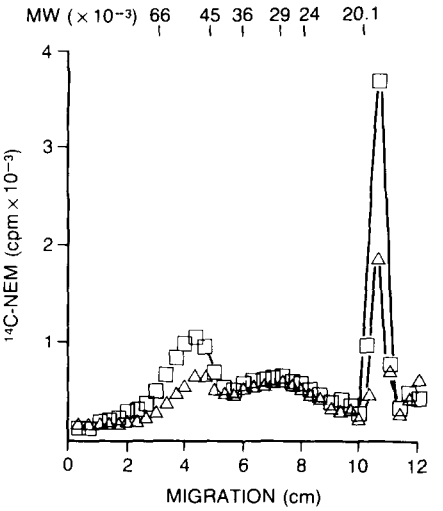

Fig. 3. Differential labelling of externally oriented cysteine residues of band 4.5 preparation. Externally located sulfhydryl groups in intact erythrocytes were masked with PCMBS prior to reacting the cells with unlabelling *N*-ethylmaleimide as described in Materials and Methods. Band 4.5 was isolated from these cells (□) and from cells that were not masked with PCMBS prior to the *N*-ethylmaleimide incubation (△). After removal of PCMBS, the band 4.5 preparations were labelled with *N*-ethyl[^{14}C]maleimide ([^{14}C]-NEM), cleaved with trypsin, and analyzed electrophoretically as in Fig. 1. The radioactivity in each slice is shown.

similar to those presented in Fig. 1, with labelling in the residual uncleaved band 4.5, and in the 30 and 19 kDa bands. The total amount of *N*-ethyl[^{14}C]maleimide labelling in Band 4.5 preparation from the protected cells is substantially higher (39%), than in the controls. Protection is evident in uncleaved band 4.5 and in the 19 kDa band. In each, there is about twice as much labelling as in the control. On the other hand, no protection is evident in the broad 30 kDa band.

The mold metabolite cytochalasin B is a potent competitive inhibitor of hexose transport that binds tightly to reconstituted band 4.5 ($K_d = 1.5 \cdot 10^{-7}$ M, Ref. 17). It can protect against inhibition by alkylating agents such as membrane-impermeant maleimides [22,23], *N*-ethylmaleimide [22,31] and fluorodinitrobenzene [32] in intact erythrocytes. It seemed likely, therefore, that cysteine residues of native band 4.5 might be modified and thereby protected against reaction with *N*-ethylmaleimide by cytochalasin B. Cytochalasin E, a structurally similar compound that does not

TABLE I

TOTAL AND REACTIVE CYSTEINE RESIDUES OF BAND 4.5

SDS-denatured and native reconstituted preparations of Band 4.5 were reacted with *N*-ethyl[^{14}C]maleimide and cleaved with trypsin as described in Materials and Methods. Values shown are the mean ± the standard deviation of the experimental sampling, with n being the number of experiments performed.

Total number of cysteine residues per polypeptide (SDS-denatured preparations)		
50 kDa intact protein	5.22 ± 0.11	$n = 4$
30 kDa tryptic fragment	2.83 ± 0.15	$n = 3$
19 kDa tryptic fragment	2.42 ± 0.08	$n = 3$
Number of reactive cysteine residues per polypeptide (Native, reconstituted preparations)		
50 kDa intact protein	2.88 ± 0.28	$n = 3$
30 kDa tryptic fragment	1.85 ± 0.21	$n = 3$
19 kDa tryptic fragment	1.03 ± 0.18	$n = 3$

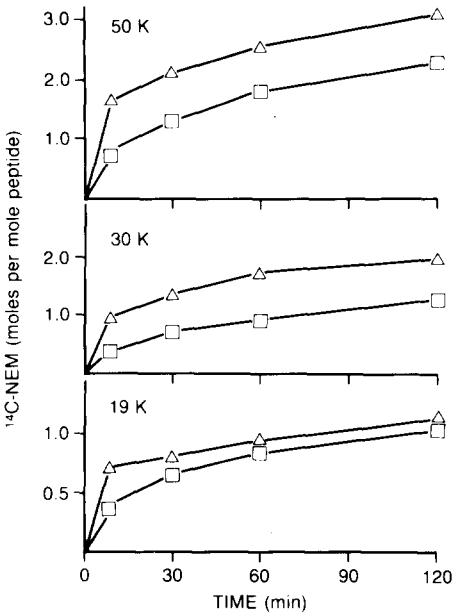

Fig. 4. Protection by cytochalasin B against reaction of *N*-ethylmaleimide with the band 4.5 preparation. Vesicles containing 'purified' peptide were incubated with 15 mM *N*-ethyl[^{14}C]maleimide ([^{14}C]-NEM) in the presence of 50 μM cytochalasin B (□) or cytochalasin E (△) for the times shown. The amount of radioactive *N*-ethylmaleimide in the portions of electrophoretograms corresponding to the intact protein (upper panel), the 30 kDa tryptic fragment (middle panel) and the 19 kDa fragment (lower panel) was determined as in Fig. 1.

bind to the transporter [33], was used as a control to eliminate non-specific effects. From Fig. 4, it is evident that cytochalasin E does not modify the *N*-ethylmaleimide labelling of band 4.5. The time-course (upper curve of Fig. 4) is virtually the same as that in Fig. 2 (no cytochalasin E), with reaction occurring with about three ligands per mole of peptide by two hours. In agreement with data in Table I, in trypsin-treated preparations two of the ligands are located in the 30 kDa and one in the 19 kDa fragments.

In contrast to cytochalasin E, cytochalasin B substantially diminished the labelling with *N*-ethyl[^{14}C]maleimide. In uncleaved band 4.5, protection was afforded throughout the 2 h period of the experiment, mounting to about 0.8 moles per mole of peptide. Most of the prolonged protection was accounted for by an *N*-ethylmaleimide-binding site in the 30 kDa fragment (Fig. 4, middle panel). It is evident, however, that some transient protection, for 10 to 20 min, was also conferred on a site in the 19 kDa fragment (Fig. 4, lower panel: note that the scale in this case is expanded by a factor of two in order to illustrate the effect). This transient protection was somewhat more pronounced with a lower concentration of *N*-ethylmaleimide (2 mM, data not shown).

Discussion

Sulfhydryl-modifying reagents inhibit transport [18–23], and in the case of those that are impermeant, inhibition has been attributed to exofacial sulfhydryl groups [19,22,23]. The maleimides, which can form covalent bonds with sulfhydryl groups, bind to band 4.5 [22,23] a component with which sugar transport has been identified [7–11]. One of the important links connecting transport to a band 4.5 component is the competitive transport inhibitor cytochalasin B, which also binds specifically to band 4.5 peptide [11]. Cytochalasin B also protects the transport system against inhibitory effects of the maleimides [22,23,31,32], and in the case of one particular impermeant maleimide, protection is afforded against its binding to band 4.5 [23].

Band 4.5 is the designation given to a broad diffusely staining zone on SDS-acrylamide gels, with molecular weight ranging from 44000 to 77000 [34]. With appropriate procedures it has been resolved into numbers of discrete as well as diffuse stained bands involving four families of peptides [35]. Functionally, it is associated not only with sugar transport, but with nucleotide transport as well [36]. Clearly, it contains a mixture of peptides. The sugar transport protein is a major component of band 4.5. It has been isolated in relatively high yield [12,17] with a purity of about 70 to 90%, based on the number of cytochalasin B binding sites per mole of peptide (assuming one binding site per peptide) [17]. After SDS-polyacrylamide gel electrophoresis, the 'purified' peptide resembles band 4.5, with a diffused stained band of M_r 50000 to 60000. The spread in molecular weight appears to be due to carbohydrate heterogeneity because after treatment with endoglycosidase F to remove most of the carbohydrate, all of the stainable peptide, except for minor components, is found as a sharp

band of 46 kDa [15,29], which also contains the cytochalasin B binding sites. It can be presumed to be largely the peptide backbone of the sugar transport protein. After trypsin cleavage the cytochalasin B binding sites are located in the 19 kDa fragment indicating that it is the transport protein that is cleavable [12,15].

Not much is known about the topology of the transport protein in the bilayer. As noted above it has at least one exofacial sulfhydryl group reactive with impermeant agents; the carbohydrate attachment site is also exofacial [37]; a trypsin cleavage site is exposed on the cytoplasmic side of the membrane [12–14]; and the cytochalasin B binding site is also thought to be located on the cytoplasmic side based on evidence from studies of inhibition kinetics [38]. The present paper extends the knowledge of topology by investigating the reaction of *N*-ethylmaleimide with binding sites in denatured and 'native' sugar transporter and by determining their distribution between the two fragments resulting from limited trypsin hydrolysis. A similar analysis is made for *N*-ethylmaleimide-binding sites modulated by cytochalasin B.

The sugar transporter is reported to have five cysteine residues based on analysis of amino-acid composition [17]. This value was confirmed by specific sulfhydryl titration with DTNB (4.7 ± 0.25). *N*-Ethylmaleimide can potentially react with ligands other than sulfhydryl groups, although under the conditions used, extensive reaction with other ligands is unlikely [16]. Because the *N*-ethylmaleimide value (5.22 ± 0.11) is not substantially higher than those obtained by specific procedures the *N*-ethylmaleimide reaction with ligands other than sulfhydryl could constitute only a minor fraction.

As reported previously [12–14], trypsin treatment of membranes (ghosts or reconstituted vesicles) produces a single cleavage, giving rise to two peptide fragments of 30 and 19 kDa (Fig. 1). In the case of the reconstituted vesicles, as also reported previously [11,12] the cleavage is incomplete. In the present experiments, based on the distribution of *N*-ethylmaleimide between uncleaved and cleaved peptide the maximal hydrolysis was 69%. This value is in essential agreement with that previously reported (75%) using covalently linked cytochalasin B as a marker [12]. To explain these findings, it is assumed that in a fraction of the vesicles (25 to 30%) the reconstituted peptide is in the right-side-out orientation, resistant to cleavage by a variety of proteolytic enzymes, including trypsin [12–14]. On the other hand, the cytoplasmic side of the peptide is susceptible to proteolytic cleavage, so that 70 to 75% of the vesicles with peptide in the inside-out orientation give rise to cleavage products. Although all five sulfhydryl ligands react with *N*-ethylmaleimide in denatured peptide, only three appear to be reactive in the native (reconstituted) preparation (Figs. 2 and 4, Table I), and the reaction is slower. Because *N*-ethylmaleimide penetrates rapidly [20], the crypticity of two residues and the slower reaction rate can be attributed to the protein's native conformation in the bilayer, which might influence the accessibility of the sulfhydryl ligands. The cryptic groups, for example, may be within the interior of the bilayer. Band 3 the anion transport protein, for example, also has an *N*-ethylmaleimide-cryptic sulfhydryl group in its membrane-crossing domain [39].

Treatment of *N*-ethylmaleimide-labelled 'native' reconstituted protein with trypsin indicates that the 30 kDa fragment contains two and the 19 kDa fragment, one *N*-ethylmaleimide-reactive group (Fig. 4, Table I). Given that these fragments contain three and two groups, respectively, in the denatured peptide (see above), each fragment must contain one cryptic group.

The impermeant sulfhydryl reagent, PCMBS [25], 'protects' one group in the 19 kDa fragment against *N*-ethylmaleimide interaction, but affords no protection to groups in the 30 kDa fragment (Fig. 3). Thus the single, non-cryptic sulfhydryl group of the 19 kDa fragment is apparently exofacial and must be functionally important (inhibition results from its interaction with PCMBS [19], or impermeant maleimides [22,23]).

The protection afforded by PCMBS in the experiment of Fig. 3 was incomplete, about 50%. This finding is not surprising because the PCMBS interaction is reversible whereas the *N*-ethylmaleimide interaction is covalent. The degree of protection should therefore decrease with time, as the irreversible reaction proceeds.

Although PCMBS affords no protection to *N*-ethylmaleimide-reactive groups in the 30 kDa frag-

ment, some *N*-ethyl[^{14}C]maleimide-labelling occurs, despite the fact that in the course of the procedure, 'non-protected' sites are exposed to reaction to non-radioactive *N*-ethylmaleimide prior to application of labelled *N*-ethylmaleimide (see Materials and Methods). It is evident therefore that the treatment of intact cells with nonradioactive *N*-ethylmaleimide did not result in complete reaction. As noted above the 30 kDa fragment has two sulfhydryl groups reactive with *N*-ethylmaleimide in 'native' transporter in the reconstituted system. Neither appears to be exofacial based on failure of PCMBS to protect. Their failure to completely react with *N*-ethylmaleimide in the intact cell may be due to their location on the cytoplasmic side of the membrane, where protection by hemoglobin could occur. Hemoglobin is rich in sulfhydryl groups and would tend to bind *N*-ethylmaleimide as it penetrates. The final labelling with *N*-ethyl[^{14}C]maleimide was done using reconstituted vesicles, in which virtually no hemoglobin is present to exert protective action, so labelling would occur.

Cytochalasin B, a competitive inhibitor of sugar transport protects the transporter against interaction and inhibition by *N*-ethylmaleimide or impermeant derivatives [22,23,31]. In the intact 'native' transporter cytochalasin B prevents the binding of about 1 mole of *N*-ethylmaleimide per mole of peptide (Fig. 4). Most of the continuing protection can be attributed to a binding group on the 30 kDa fragment, but there is also transient protection of the single-reactive exofacial group of the 19 kD fragment. The latter can account for the protection by cytochalasin B against effects and binding of impermeant maleimides [22,23]. The modulation by cytochalasin B of another sulfhydryl group located on the 30 kDa fragment and presumably cytoplasmic in location is consistent with the report that internal as well as exofacial sulfhydryl groups may be the sites of inhibition by maleimides [20].

Based on the above findings and on previously reported information, a proposed arrangement of band 4.5 in the erythrocyte membrane is shown in Fig. 5. As shown previously [12–14], limited tryptic digestion of the protein at the membrane cytoplasmic face produces two major fragments, a peptide of approx. 30 kDa that contains the

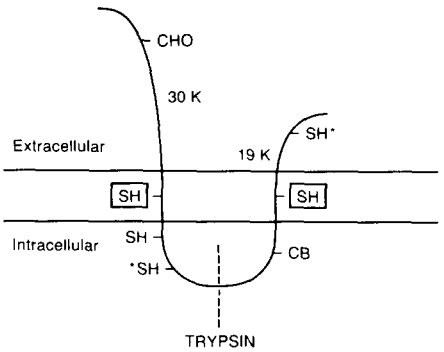

Fig. 5. A proposed arrangement of sugar transport protein in the human erythrocyte membrane. The two membrane-spanning fragments of the protein along with the site of tryptic cleavage are shown. Also represented are the carbohydrate attachment site (CHO), the proposed location of the cytochalasin B binding site (CB), and the protein's cysteine residues (SH). The cryptic sulfhydryl groups are shown in a box and those groups protected by cytochalasin B are designated by *.

carbohydrate moiety, and a peptide of 19 kDa that bears the site photolabelled with cytochalasin B, a site assigned to the cytoplasmic face, based on studies of inhibition kinetics [38]. The present study demonstrates that the 19 kDa fragment contains two of the five cysteine residues of band 4.5, one of which is reactive with *N*-ethylmaleimide in the reconstituted system, and is located at the outside face of the membrane. Thus this fragment must cross the bilayer. The 30 kDa fragment contains the remaining three sulfhydryl groups, two of which are reactive in the native protein. Because this fragment is produced by cleavage at the membrane cytoplasmic face and because it contains the polypeptide's carbohydrate, which is located at the cell's exterior [37], it must also span the membrane. Its two *N*-ethylmaleimide-reactive groups are placed on the cytoplasmic side because they are not protected by the impermeant agent PCMBS, and because they appear to be afforded some protection by hemoglobin in the intact cell. The two cryptic groups, one in each fragment, are tentatively placed within the bilayer. This location is not definitively established, but it could account for crypticity, and it is consistent with the finding that lipid-soluble maleimides can reach inhibitory sites inaccessible to water soluble maleimides [20]. The model is presented as a beginning in understanding the topology of the sugar transport pro-

tein. As in the case of other transport proteins later models will prove to be far more complicated.

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References

1 Jung, C.Y. (1975) in The Red Blood Cell, Vol. 2 (Surgenor, D. MacN., ed.), pp. 705–751, Academic Press, New York
2 Baldwin, S.A. and Lienhard, G.E. (1981) Trends Biochem. Sci. 6, 208–211
3 Jones, M.N. and Nickson, J.K. (1981) Biochim. Biophys. Acta 650, 1–20
4 Jones, M.N. and Nickson, J.K. (1980) FEBS Lett. 115, 1–8
5 Mullins, R.E. and Langdon, R.G. (1980) Biochemistry 19, 1205–1212
6 Shelton, R.L. and Langdon, R.G. (1983) Biochim. Biophys. Acta 733, 25–33
7 Baldwin, S.A. and Lienhard, G.E. (1980) Biochem. Biophys. Res. Commun. 94, 1401–1408
8 Lienhard, G.E., Gorga, F.R., Orasky, J.E. and Zoccoli, M.A. (1977) Biochemistry 16, 4921–4926
9 Kashahara, M. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7384–7390
10 Wheeler, T.J. and Hinkle, P.C. (1981) J. Biol. Chem. 256, 8907–8914
11 Baldwin, S.A., Baldwin, J.M., Gorga, F.R. and Lienhard, G.E. (1979) Biochim. Biophys. Acta 522, 183–188
12 Deziel, M.R. and Rothstein, A. (1984) Biochim. Biophys. Acta 776, 10–20
13 Cairns, M.T., Elliot, D.A., Scudder, P.R. and Baldwin, S.A. (1984) Biochem. J. 221, 179–188
14 Shanahan, M.F. and D'Artel-Ellis, J. (1984) J. Biol. Chem. 259, 13878–13884
15 Klip, A., Deziel, M. and Walker, D. (1984) Biochem. Biophys. Res. Commun. 122, 218–224
16 Means, G.E. and Feeney, R.E. (1971) Chemical Modification of Proteins, Holden-Day, San Francisco
17 Baldwin, S.A., Baldwin, J.M. and Lienhard, G.E. (1982) Biochemistry 21, 3836–3842
18 Dawson, A.C. and Widdas, W.F. (1963) J. Physiol. 168, 644–659
19 Van Steveninck, J., Weed, R.I. and Rothstein, A. (1965) J. Gen. Physiol 48, 617- 632
20 Smith, R.P.P. and Ellman, G.L. (1973) J. Membrane Biol. 12, 177–188
21 Bloch, R. (1974) J. Biol. Chem. 249, 1814–1822
22 Batt, E.R., Abbott, R.E. and Schachter, D. (1976) J. Biol. Chem. 251, 7184–7190
23 Roberts, S.J., Tanner, M.J.A. and Denton, R.M. (1982) Biochem. J. 205, 139- 145
24 Steck, T.L. and Yu, J. (1973) J. Supramol. Struct. 1, 220–232
25 Rothstein, A. (1981) in The Function of Red Blood Cells: Erythrocyte Pathobiology (Wallach, D.F.H., ed.), pp. 105–131, Allen R. Liss, New York
26 Cabantchik, Z.I., Knauf, P.A. and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239–302
27 Laemmli, U.K. (1970) Nature 227, 680–685
28 Lowry O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
29 Lienhard, G.E., Crabb, J.H. and Ransome, K.J. (1984) Biochim. Biophys. Acta 769, 404–410
30 Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70–76
31 Lefevre, P.G., D'Angelo, G. and Masiak, S.J. (1975) Fed. Proc. 34, 238
32 Krupka, R.M. and Deves, R. (1980) J. Biol. Chem. 255, 2051–2055
33 Rampal, A.L., Pinkofsky, H.B. and Jung, C.Y. (1980) Biochemistry 19, 679–683
34 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
35 Whitfield, C.F., Coleman, D.B., Kay, M.M., Schiffer, K.A. Miller, J. and Goodman, S.R. (1985) Am. J. Physiol. 248, C70–C79
36 Jarvis, S.M. and Young, J.D. (1981) Biochem. J. 194, 331–339
37 Gorga, F.R., Baldwin, S.A. and Lienhard, G.E. (1979) Biochem. Biophys. Res. Commun. 91, 955–961
38 Deves, R. and Krupka, R.M. (1978) Biochim. Biophys. Acta 510, 339–348
39 Solomon, A.K., Chasan, B., Dix, J.A., Lukacovic, M.F., Toon, M.R. and A.S. Verkman (1983) Ann. N.Y. Acad. Sci. 414, 97–124