Interaction of a permeant maleimide derivative of cysteine with the erythrocyte glucose carrier

Differential labelling of an exofacial carrier thiol group and its role in the transport mechanism

James M. MAY

Diabetes Research and Training Center, Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232–2230, U.S.A.

S-(Bismaleimidomethyl ether)cysteine (Cys-Mal) was synthesized as a probe for reactive thiol groups on the erythrocyte glucose carrier. Although Cys-Mal entered cells, its reaction with intracellular GSH prevented alkylation of endofacial membrane proteins, limiting its effect to the cell surface at concentrations below 5 mM. Cys-Mal irreversibly inhibited hexose transport half-maximally at 1.5 mM by decreasing the maximal rate of transport, with no effect on the affinity of substrate for the carrier. Reaction occurred with the outward-facing form of the carrier, but did not affect the ability of the carrier to change orientation. In intact cells, several exofacial proteins were labelled by [³⁵S]Cys-Mal, including the band-4.5 glucose carrier, the labelling of which occurred on a single site sensitive to transport inhibitors. The reactive exofacial group was a thiol group, since both transport inhibition and band-4.5 labelling by Cys-Mal were abolished by the thiol-specific and impermeant compound 5,5'-dithiobis(2-nitrobenzoic acid). Selectivity for carrier labelling in cells was increased by a double differential procedure, which in turn allowed localization of the exofacial thiol group to the *M_r* 18 000–20 000 membrane-bound tryptic carrier fragment. In protein-depleted ghosts the exofacial thiol group was preferentially labelled at low concentrations of [³⁵S]Cys-Mal, whereas with the reagent at 10 mM the *M_r* 26 000–45 000 tryptic carrier fragment was also labelled. Cys-Mal should be useful in the study of carrier thiol-group location and function.

INTRODUCTION

The presence of a reactive outwardly exposed thiol group required for transport on the human erythrocyte glucose carrier was first suggested by VanSteveninck *et al.* [1], using the poorly permeant compound *p*-chloromercuribenzenesulphonate (PCMBS), which has high specificity for thiols [2]. Most subsequent studies of this exofacial carrier thiol have employed impermeant maleimides [3–8], which have substantial, although not complete, thiol specificity [9–11]. These agents irreversibly inhibit hexose transport and label the carrier protein in intact cells, although the selectivity of labelling is low [3–6,8]. Recent evidence suggests that the exofacial thiol group is exposed only when the carrier is in a conformation with the substrate-binding site facing outward [12,13], according to the one-site or alternating conformation model of transport [14,15]. Thus preincubation with maltose, an impermeant competitive transport inhibitor, accelerates transport inactivation by impermeant reagents, presumably by holding carriers in an outward-facing conformation with more thiol groups available for reaction [7,8,13]. On the other hand, cytochalasin B, which binds to the inward-facing carrier [16], protects from transport inactivation by pulling carriers to an inward-facing conformation [4–8,13]. The protective effect of cytochalasin B has in fact been used in differential labelling protocols to improve the selectivity of labelling of the hexose carrier with such reagents [4,5,8].

However, use of existing reagents is limited by difficulty in their preparation [5,6,8], or by the expense of the radioactive form [3,6]. For these reasons, and to obtain a derivative which might have effects contrasting with those of available probes, I have synthesized *S*-(bismaleimidomethyl ether)-L-cysteine (Cys-Mal). Although it entered erythrocytes, Cys-Mal irreversibly inhibited hexose uptake by reacting with the exofacial-carrier thiol group. With the use of differential labelling techniques it was possible to label selectively the exofacial thiol group in intact cells and protein-depleted ghosts, and to localize the endofacial carrier thiol(s) to the *M_r* 23 000–40 000 tryptic fragment of the carrier.

EXPERIMENTAL

Materials

Radionuclides were obtained as follows: 3-*O*-[methyl-¹⁴C]methylglucose (40 Ci/mol) from ICN, [4-³H]cytochalasin B (15 Ci/mmol) from New England Nuclear, and L-[³⁵S]cysteine (1.1 Ci/mmol) from Amersham International.

Synthesis of Cys-Mal

Cys-Mal was synthesized by the method of Abbott & Schachter [3] for glutathione-maleimide-I (GS-Mal). Briefly, 0.1 g (0.424 mmol) of bismaleimidomethyl ether [17] was dissolved in 19 ml of water at 70 °C, and to this

Abbreviations used: PCMBS, *p*-chloromercuribenzenesulphonate; Cys-Mal, *S*-(bismaleimidomethyl ether)-L-cysteine; GS-Mal, glutathione maleimide-I; PBS, phosphate-buffered saline (12.5 mM-sodium phosphate/150 mM-NaCl, pH 7.4); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

was added with stirring a solution of 0.17 g (0.14 mmol) of L-cysteine base in 1 ml of water over 10 min. The reaction solution was allowed to cool and extracted five times with 100 ml of ethyl acetate. Residual ethyl acetate in the aqueous fraction was removed under a stream of nitrogen, and the solution freeze-dried. The fluffy white powder was used without further purification in most studies. T.l.c. in propan-2-ol/ethyl acetate/water (7:2:1, by vol.) showed no evidence of bismaleimidomethyl ether or hydrophobic conjugates in the preparation. Measurement of thiol reactivity as previously described [6] showed 95–100% of the expected reactivity on the basis of weight. The derivative was stable by this assay for at least 4 months when stored dessicated at -20°C . A small sample was further purified by column chromatography on Sephadex G-10 [3] and submitted to ^1H n.m.r. and elemental analysis. Significant features of ^1H n.m.r. spectra in $^2\text{H}_2\text{O}$: δ (chemical shift in p.p.m.) 3.2–3.4 (2H, series of multiplets, CH_2S), 3.9 (1H, t, proton on cysteine chiral carbon), 4.9 (4H, s, CH_2OCH_2), 6.8 (2H, s, maleoyl protons). Found: C, 41.4; H, 4.5; N, 11.0, S, 9.3%; $\text{C}_{13}\text{H}_{15}\text{N}_3\text{O}_7\text{S}_1\text{H}_2\text{O}$ (375.2) requires C, 41.6; H, 4.5; N, 11.2; S, 8.5%.

Radioactive Cys-Mal was prepared by adding 2 mg of bismaleimidomethyl ether in 0.4 ml of distilled water at 70°C to 500 μCi of L- ^{35}S cysteine (0.05 ml in aqueous 20 mM-potassium acetate containing 5 mM-dithiothreitol, as obtained from the manufacturer). The reaction was allowed to continue for 30 min at room temperature, whereupon 1 ml of water was added and the solution extracted four times with 2 ml of ethyl acetate. Residual ethyl acetate was removed from the aqueous phase under a stream of nitrogen, and the preparation was stored in small aliquots at -70°C until use. Over 90% of the radioactivity was found in the aqueous fraction. The thiol reactivity of ^{35}S Cys-Mal was confirmed by using an assay involving thiopropyl-Sepharose as previously described [6] and was stable frozen for at least 3 weeks. Radioactive ^{35}S Cys-Mal was chemically diluted with unlabelled Cys-Mal to the indicated specific radioactivity before use.

Erythrocyte preparation and assays

Erythrocytes were prepared, and uptake of 3-*O*-methylglucose (5.2 μM , 40 Ci/mol) was measured in 12.5 mM-sodium phosphate buffer, pH 7.4, containing 150 mM-NaCl (PBS), as previously described [6]. The kinetics of hexose uptake (0.5–4 mM-hexose) were also measured as previously described [7]. Concentrations of maleimide reagents are expressed as the initially added extracellular concentration. Erythrocyte GSH content was measured by the method of Hissen & Hilf [18]. Glyceraldehyde-3-phosphate dehydrogenase [GAPDH; D-glyceraldehyde-3-phosphate:NAD $^{+}$ oxidoreductase (phosphorylating), EC 1.2.1.12] activity in leaky ghosts was determined by a spectrophotometric method [19]. Binding of ^3H cytochalasin B to intact erythrocytes was measured as previously described [7]. In experiments designed to measure the equilibrium dissociation constant and total specific binding in intact cells, erythrocytes in PBS at a 10% haematocrit were incubated with 62.5 nCi of ^3H cytochalasin B, 10 μM -cytochalasin E and one of several concentrations of unlabelled cytochalasin B (10, 62.5, 125, 250, and 3000 nM) for 15 min at 37°C before termination of the assay. The data obtained with different concentrations of cytochalasin B were subjected to Scat-

chard analysis as modified by Rosenthal [20]. Correction was made for non-specific binding measured in the presence of 3000 nM-cytochalasin B. Maltose inhibition of the binding of a low concentration of cytochalasin B (10 nM) was performed as previously described [7], using maltose concentrations of 0, 30, 60, 120, and 210 mM.

Erythrocyte lysis and membrane protein electrophoresis

Hypo-osmotic lysis and preparation of white ghosts was performed by the method of Fairbanks *et al.* [21]. Protein-depleted ghosts were prepared as described by Gorga & Lienhard [15]. The membranes were stored at -20°C until subjected to SDS/polyacrylamide-gel electrophoresis [22] with several modifications [8]. Samples were counted for radioactivity until at least 1000 disintegrations had accumulated in the gel slices of interest. Calculations of the number of labelled sites per cell were based on the measured specific radioactivity of ^{35}S Cys-Mal, an estimate of 1.39×10^6 cells/ μg of leaky-ghost membrane protein applied to gels [23], and the total radioactivity in the band-4.5 region on gels corrected for environmental background (usually 20–30 d.p.m.).

Data analysis

Data are shown as means $\pm$ S.E.M. from the indicated number of experiments. Statistical comparisons were made by using Student's *t* test for paired values.

RESULTS AND DISCUSSION

Transport inhibition by Cys-Mal

Incubation of erythrocytes with 5 mM-Cys-Mal resulted in a time-dependent inhibition of 3-*O*-methylglucose uptake, which was complete by about 30 min (results not shown). After a 30 min incubation, transport was progressively inhibited by increasing concentrations of Cys-Mal, although about 30% of control transport was unaffected, even at Cys-Mal concentrations of 10 mM or greater (Table 1). Inhibition to half of the maximal effect observed occurred at a Cys-Mal concentration of about 1.5 mM. The potency of GS-Mal was similar to that of Cys-Mal under these conditions (Table 1). The V_{max} for inhibition of 3-*O*-methylglucose entry was decreased by 5 mM-Cys-Mal from 4.5 ± 0.9 to $2.0 \pm 0.3 \mu\text{mol}^{-1} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$ ($P < 0.05$, $n = 3$), whereas there was no effect on the transport K_m (control, 0.8 ± 0.1 mM; Cys-Mal, 0.7 ± 0.2 mM).

Reaction of Cys-Mal with the exofacial carrier thiol group

If the transport inhibition caused by Cys-Mal is due to reaction with an exofacial carrier thiol, then reversible blockade of this group should prevent the transport inhibition induced by Cys-Mal. This was achieved with the use of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), an impermeant thiol-specific reagent which inhibits hexose transport by reacting in a thiol-reversible manner with the exofacial thiol group [24]. Whereas the transport inhibition induced by DTNB is not reversed by washes with PBS [24], incubation and washes in a cysteine-containing buffer did reverse its inhibition of transport (Table 1). Pretreatment of cells with 4 mM-DTNB prevented the inhibitory effects of 5 mM-Cys-Mal under these conditions (Table 1), in support of the hypothesis that the transport inhibition caused by Cys-Mal is related to its reaction with the exofacial carrier thiol group.

Table 1. Transport inhibition by Cys-Mal

(a) Dose-response of transport inhibition. Erythrocytes in 0.8 ml of PBS at a 20% haematocrit were incubated with the indicated concentration of Cys-Mal or GS-Mal at 37 °C for 30 min before three washes by centrifugation in 5 ml of PBS at 37 °C. After the last wash, the suspension was restored to the original volume and the transport assay performed in triplicate. (b) DTNB protection from Cys-Mal-induced transport inhibition. Erythrocytes as in (a) were incubated without or with 4 mM-DTNB for 30 min at 37 °C, followed, where indicated, by addition of Cys-Mal to a final concentration of 5 mM. After an additional 30 min at the same temperature, all samples were diluted with 6 vol. of PBS containing 10 mM-cysteine and incubated an additional 10 min at 37 °C, centrifuged, and the supernatant removed. The cysteine incubation and wash were repeated once more before a final wash in 6 vol. of PBS and the transport assay. (c) Effects of competitive inhibitors. Erythrocytes were incubated by 10 min at 37 °C with the indicated inhibitor, followed by addition of 2 mM-Cys-Mal for 30 min at the same temperature. The cells were washed twice in 5 ml of PBS containing bovine serum albumin (20 mg/ml) (to aid in the removal of the cytochalasins) and twice more in PBS alone before readjustment to the original volume and assay of hexose transport. Data are from *n* experiments expressed relative to an untreated control sample. An asterisk indicates *P* < 0.02 compared with other treatments in the series.

Series	Conditions	Transport rate (% of control)	<i>n</i>
<i>a</i>	Cys-Mal		
	0.6 mM	80 ± 2	5
	1.3 mM	67 ± 5	
	2.5 mM	59 ± 1	
	5.0 mM	48 ± 4	
	10 mM	33 ± 4	
	20 mM	32 ± 2	
	GS-Mal 5 mM	53 ± 4	
<i>b</i>	DTNB alone	93 ± 10	6
	Cys-Mal alone	51 ± 8*	
	DTNB + Cys-Mal	94 ± 7	
<i>c</i>	Sucrose (120 mM)	62 ± 4	6
	Maltose (120 mM)	45 ± 4*	
	Cytochalasin E (6.5 μM)	64 ± 6	4
	Cytochalasin B (6.5 μM)	91 ± 14*	

Cys-Mal and changes in carrier conformation

The effects of carrier conformational changes on irreversible transport inhibition by Cys-Mal are shown in Table 1. A high concentration of maltose enhanced the transport inhibition produced by a submaximal concentration of Cys-Mal. On the other hand, cytochalasin B protected the carrier from irreversible inhibition by Cys-Mal. On the basis of results with other agents [7,8,13], these data suggest reaction of Cys-Mal with the outward-facing carrier conformation, in accord with the one-site model of transport.

Conversely, reaction of Cys-Mal with the carrier might also be expected to affect carrier conformation. This possibility was tested by treating intact cells for 30 min at 37 °C with 5 mM-Cys-Mal, washing, and assaying equilibrium cytochalasin B binding. Cytochalasin B binds only to the inward-facing carrier [16], so that any

tendency of Cys-Mal to 'lock' carriers in an outward-facing conformation would decrease cytochalasin B binding. However, in four such experiments Cys-Mal had no effect on either the equilibrium dissociation constant for cytochalasin B (control: 120 ± 4 nM; Cys-Mal: 140 ± 12 nM), or on total specific binding (control: 4.5 ± 0.2 nmol/ml of packed cells; Cys-Mal: 4.5 ± 0.2 nmol/ml of packed cells). Assuming 10^{10} cells/ml of packed cells, specific cytochalasin B binding occurred with 270000 sites per cell. Additionally, Cys-Mal did not significantly affect the ability of maltose outside cells to inhibit the binding of cytochalasin B to the inward-facing carrier. In seven experiments the apparent *K_i* for maltose was 28 ± 3 mM in untreated cells and 39 ± 4 mM in cells treated with Cys-Mal. Sucrose alone at 210 mM had no effect on cytochalasin B binding. Similar effects were also observed with DTNB [24], but contrast with those of GS-Mal [7] and maleimide derivatives of glucosamine [8], which 'lock' the carrier in an outward-facing conformation unable to bind cytochalasin B. Such variable effects of agents with different size and charge suggest that the exofacial thiol group may be located in a region sensitive both to substrate translocation and to carrier conformational changes, and that it might be a good site for study of the carrier mechanism.

Cellular penetrance of Cys-Mal

In order to confirm an exofacial site of transport inhibition by Cys-Mal, its ability to enter erythrocytes was assessed. Increasing extracellular concentrations of

Fig. 1. Cellular penetrance of Cys-Mal

Erythrocytes (0.2 ml) at a 20% haematocrit were incubated with the indicated concentration of Cys-Mal for 30 min at 37 °C, washed three times by centrifugation in 5 ml of PBS at 37 °C, and resuspended to the initial volume for assay of intracellular GSH content (in duplicate) (●). For assay of GAPDH activity (○), 1.6 ml of 20% erythrocytes were incubated as above, washed four times by centrifugation in 10 ml of PBS at 37 °C, subjected to hypo-osmotic lysis, and enzyme activity was measured in leaky ghost membranes (corrected for protein recovery). Data are from four experiments with each assay and are expressed as a percentage of untreated-control value.

Cys-Mal caused a linear fall in GSH content, which was half-maximal at 5 mM reagent (Fig. 1). Depletion of GSH by Cys-Mal was not affected by 100 μ M-phloretin or 50 μ M-cytochalasin B (not shown). The cellular penetration of Cys-Mal was also assessed by measurement of GAPDH activity in leaky ghosts prepared from cells which had been pretreated with several concentrations of Cys-Mal. No significant effect of Cys-Mal on enzyme activity was evident at concentrations up to 20 mM-reagent, although there was a definite fall in some experiments at the highest concentration (Fig. 1). On the other hand, preincubation under the same conditions with 0.3 mM-*N*-ethylmaleimide resulted in a 35% fall in activity of the enzyme ($P < 0.01$; results not shown). Thus any Cys-Mal entering cells is consumed by GSH, so that reaction with membrane-bound GAPDH (and presumably other cytosolic membrane proteins) is delayed until GSH stores are depleted. In spite of the fact that Cys-Mal enters erythrocytes, its reaction with membrane proteins appears limited to portions exposed on the exofacial cell surface.

$[^{35}\text{S}]\text{Cys-Mal}$ labelling of intact cells

Electrophoretic profiles of leaky erythrocyte ghost protein prepared from cells treated with $[^{35}\text{S}]\text{Cys-Mal}$ showed labelling of several peaks (Fig. 2a). In three such experiments, the band-4.5 peak [25] contained an average of $66 \pm 4\%$ of total radioactivity in peaks of interest. Under these conditions, band-4.5 labelling using increasing concentrations of $[^{35}\text{S}]\text{Cys-Mal}$ was saturable, with a half-maximal effect derived from non-linear regression [26] at about 1 mM-reagent, corresponding to the dose-response relationship of transport inhibition (Table 1). The calculated maximal number of band-4.5 sites was about 180 000/cell, in agreement with the results from other methods [27–29]. This suggests that, under these conditions, at most one site in band 4.5 is labelled.

No labelling of proteins located exclusively on the cytoplasmic face of the membrane (e.g. GAPDH, spectrin or actin) was evident in any of several experiments performed at $[^{35}\text{S}]\text{Cys-Mal}$ concentrations up to 4.8 mM, although these proteins were present in the Coomassie Blue-stained gels (results not shown). Thus only exofacially exposed proteins were labelled in intact cells.

In this and two other experiments, the labelling of the band-4.5 peak was selectively suppressed about 60–70% by preincubation with 50 μ M-cytochalasin B compared with 50 μ M-cytochalasin E, which produced results identical with those of untreated controls; results not shown (Fig. 2a). In Fig. 2(b) the presence of 100 mM-maltose before and during reaction with a relatively low concentration of $[^{35}\text{S}]\text{Cys-Mal}$ increased labelling of the band-4.5 peak by 29% compared with a sucrose control. In another experiment (not shown), band-4.5 labelling by $[^{35}\text{S}]\text{Cys-Mal}$ was doubled by maltose under these conditions. These data are in agreement with the transport studies of Table 1, indicating protection from Cys-Mal-induced transport inactivation by cytochalasin B and acceleration by maltose. Together they support the notion that the band-4.5 peak labelled by $[^{35}\text{S}]\text{Cys-Mal}$ contains the carrier protein.

Differential labelling studies

In an effort to increase the selectivity of band-4.5 labelling by Cys-Mal, a 'double' differential labelling procedure was employed that was analogous to that used

Fig. 2. Labelling of intact cells with $[^{35}\text{S}]\text{Cys-Mal}$

(a) Erythrocytes at a 50% haematocrit (1 ml) were incubated for 10 min at 37 °C with either 50 μ M-cytochalasin E (●) or -cytochalasin B (○), whereupon $[^{35}\text{S}]\text{Cys-Mal}$ (10 Ci/mol) was added to a final concentration of 1.4 mM. The incubation was continued for another 30 min, followed by four washes in 10 ml of PBS at 37 °C and preparation of leaky ghost membranes, 120 μ g of which were subjected to gel electrophoresis as described in the Experimental section. Under the conditions of (a), erythrocytes were incubated for 10 min with 120 mM-sucrose (●) or 120 mM-maltose (○) and labelled with 0.12 mM- $[^{35}\text{S}]\text{Cys-Mal}$ (109 Ci/mol). Electrophoresis was performed by using 150 μ g of leaky ghost membrane protein and 9%-(w/v)-acrylamide gels. The locations of prestained M_r markers run in adjacent lanes are indicated by arrows (the values represent $10^{-3} \times M_r$).

by Shanahan & Jacquez [30] to improve the selectivity of fluorodinitrobenzene for the carrier. Cells were first treated with cytochalasin B to protect the exofacial carrier thiol group from subsequent alkylation by 5 mM unlabelled Cys-Mal. The cells were washed free of these reagents and the carriers reoriented to an outward-facing conformation by using phloretin [7,8,13,31], followed by treatment with $[^{35}\text{S}]\text{Cys-Mal}$ in the usual fashion. A representative electrophoretic pattern is shown in Fig. 3. This procedure increased the specificity of labelling of band 4.5, such that it contained an average of $81 \pm 2\%$ of the total radioactivity in four experiments. Cells treated

Fig. 3. Differential labelling of intact erythrocytes with [^{35}S]-Cys-Mal

Erythrocytes at a 50% haematocrit were incubated for 10 min at 37 °C with either 50 μM -cytochalasin B (●) or -cytochalasin E (○), followed by addition of unlabelled Cys-Mal to 5 mM, incubation for another 30 min at the same temperature, and four washes by centrifugation in 10 vol. of PBS at 37 °C. Cells were resuspended to the original volume, treated with 1% (v/v) ethanol (○) or 100 μM -phloretin in PBS contained 1% ethanol (●) for 10 min at 37 °C, followed by addition of 1.4 mM- ^{35}S -Cys-Mal (10.3 Ci/mol) and incubation for another 30 min. Labelling was terminated by four washes in 10 vol. of PBS at 37 °C. Leaky ghosts were prepared and 120 μg of protein was subjected to gel electrophoresis on 9%-acrylamide gels. M_r marker positions are shown as in Fig. 2; 'TD' marks the position of the tracking dye.

initially with cytochalasin E instead of cytochalasin B showed labelling only in the M_r 75000–105000 peaks; labelling of the band-4.5 peak was totally suppressed, indicating the specificity of the procedure (Fig. 3). Treatment of intact cells with 6 mM-DTNB followed by the cytochalasin B/phloretin protection procedure abolished labelling of the band-4.5 carrier, confirming the thiol-group specificity of the reaction (results not shown).

Tryptic-digestion studies

Tryptic digestion of ghosts prepared from cells which had been differentially labelled with [^{35}S]Cys-Mal left only a single sharp M_r = 18000–20000 peak (Fig. 4). Further studies suggested that this fragment was derived solely from labelled band 4.5. First, in experiments performed as described in Fig. 3, in which band-4.5 labelling was ablated by omitting cytochalasin B during reaction with unlabelled Cys-Mal, so that the M_r -75000–105000 peaks were the *only* ones labelled, tryptic digestion removed essentially all radioactivity from the gels. Secondly, tryptic digestion of leaky ghosts in which the band-4.5 carrier had been photolabelled with cytochalasin B [7] also resulted in an M_r -18000–20000 tryptic fragment from labelled band 4.5, corresponding to that observed in previous studies [32–35]. Thirdly, in cells treated with the double-differential-labelling procedure having the exofacial carrier thiol selectively exposed, the presence of 100 mM-maltose during labelling with 0.3 mM- ^{35}S -Cys-Mal resulted in a doubling of label in the M_r -18000–20000 tryptic fragment, whereas 50 μM -cyto-

Fig. 4. Tryptic digestion of ghosts prepared from cells differentially labelled by Cys-Mal

Intact cells were differentially labelled by using sequential treatment with cytochalasin B and phloretin, followed by labelling with 2.5 mM- ^{35}S -Cys-Mal (13.8 Ci/mol) as described in the legend to Fig. 3. Leaky erythrocyte ghosts were prepared from these cells and resuspended to a concentration of 2–4 mg/ml in 5 mM-sodium phosphate buffer, pH 8.0. The sample was subdivided into equal fractions; one aliquot was incubated with the phosphate buffer (●) and the other with trypsin (1200 units/ml) (○) for 30 min at 37 °C. Digestion was terminated with the addition of soybean trypsin inhibitor to a concentration of 2300 units/ml. After another 5 min the ghosts were washed twice by centrifugation in 10 ml of ice-cold phosphate buffer at 18000 g . Electrophoresis was performed by using 120 μg of ghost protein applied to a 12%-acrylamide gel. M_r markers are shown as in Fig. 2; 'TD' marks the position of the tracking dye.

chalasin B decreased labelling of the fragment by 85%. Additionally, when correction was made for non-carrier protein lost from the membranes during tryptic digestion (Fig. 4), the labelled tryptic fragment accounts for 86% of band-4.5 labelling in three experiments, indicating no loss of labelled thiol.

Since at most one exofacial thiol group in each band-4.5 molecule was labelled, and since labelled band 4.5 was exclusively and stoichiometrically converted into the M_r -18000–20000 tryptic fragment, the exposed exofacial thiol must be on this carrier fragment. Deziel *et al.* [36] obtained indirect evidence for a single exofacial carrier thiol. In their studies, the only thiol protected by reaction of intact cells with PCMBs from subsequent exhaustive alkylation by N - ^{14}C -ethylmaleimide was in this fragment. Recently I also found similar results using a differential-labelling technique with a maleimide derivative of glucosamine [8]. According to hydropathy analysis of the primary amino acid sequence for this type of carrier [37], the most likely candidate for the exofacial thiol of the three present in the M_r -18000–20000 tryptic fragment is Cys⁴²⁹, just one amino acid away from entering the bilayer. Acknowledging the limitations of hydropathy

analysis in predicting the location of specific residues of multispanning membrane proteins [38], further studies will be necessary to confirm this location.

Differential labelling of protein-depleted ghost membranes with [35 S]Cys-Mal

In order to determine the availability of the exofacial and possibly other carrier thiols under differential-labelling conditions in which [35 S]Cys-Mal has access to both sides of the membrane, protein-depleted ghosts were subjected to the double-differential-labelling procedure at a low [35 S]Cys-Mal concentration with the electrophoretic results shown in Fig. 5(a). It is evident that several proteins were labelled under those conditions, including band 4.5. The latter contains the hexose carrier, since phloretin in the final incubation before the labelling step selectively enhanced incorporation of radioactivity into this region compared with treatment with cytochalasin B (Fig. 5a). Furthermore, in another experiment, 100 mM-D-glucose present during the labelling step also selectively decreased band-4.5 labelling by 19% compared with the same concentration of L-glucose (results not shown).

Tryptic digestion of the same labelled protein-depleted ghosts shown in Fig. 5(a) resulted in the electrophoretic pattern of Fig. 5(b), in which prominent differential effects of phloretin and cytochalasin B occurred in a sharp M_r -18000–20000 fragment, but not in the leading M_r -20000–22000 shoulder of this peak or consistently in other peaks. The identity of the M_r -20000–22000 peak is unknown, but it may correspond to an *N*-ethylmaleimide-labelled tryptic fragment of band 3 reported by Rao & Reithmeier [39] to occur in this region. The M_r -18000–20000 peak corresponds in location to that remaining after tryptic digestion of leaky ghosts prepared from intact cells which had been differentially labelled with [35 S]Cys-Mal (Fig. 4). Moreover, blockade of exofacial thiols with 5 mM-Cys-Mal before preparation of protein-depleted ghosts, differential labelling and tryptic digestion resulted in selective suppression of labelling of this peak (results not shown), further suggesting that it reflects labelling of the exofacial thiol group.

When the concentration of radioactive Cys-Mal was raised to 10 mM, differential labelling of the band-4.5 carrier of protein-depleted ghosts was evident (Fig. 6a). Further, band-4.5 labelling relative to band 3 was increased when compared with that observed at 0.4 mM-reagent (see Fig. 5a). After tryptic digestion, label was retained in both the M_r -18000–20000 fragment and in a broad M_r -26000–45000 region (Fig. 6b). Labelling of both areas followed the pattern expected for carrier labelling in that cytochalasin B in the first step of the differential-labelling protocol enhanced labelling compared with cytochalasin E (Fig. 6b). In additional experiments, the presence of either 100 mM-D-glucose or 6 mM-DTNB during the labelling step decreased the radioactivity in the two peaks (not shown), confirming their carrier origin and thiol reactivity. The apparent separation of the M_r -26000–45000 fragment into two peaks was not a consistent finding in other studies. However, this pattern has been previously observed by Cairns *et al.* [34,35] in Coomassie Blue-stained tryptic fragments of the carrier, and was attributed by them to some dimerization of the M_r -18000–20000 fragment [34]. Labelling of the M_r -26000–45000 tryptic fragment in ghosts at high (Fig. 6b) as opposed to low (Fig. 5b) [35 S]Cys-Mal

Fig. 5. Differential labelling with [35 S]Cys-Mal and tryptic digestion of labelled protein-depleted ghosts

Freshly prepared protein-depleted ghosts were resuspended to 3–4 mg/ml in 2 ml of 5 mM-sodium phosphate buffer, pH 8.0, and incubated for 5 min at 37 °C with 50 μ M-cytochalasin B. This was followed by addition of unlabelled Cys-Mal to a concentration of 5 mM and incubation for 30 min at the same temperature. The ghosts were washed three times by centrifugation at 18 000 *g* in 40 ml of ice-cold phosphate buffer and resuspended to the original volume. The ghosts were split into two equal aliquots, one of which was treated with 100 μ M-cytochalasin B (○) and the other with 100 μ M-phloretin (●) for 10 min at 37 °C. Labelling with 0.4 mM-[35 S]Cys-Mal (129 Ci/mol) was performed for 30 min at the same temperature, followed by two centrifugation washes in 40 ml of ice-cold phosphate buffer. The ghosts were again resuspended to the original volume and one aliquot from each treatment incubated without (a) or with (b) trypsin as described in the legend to Fig. 4. For each treatment 150 μ g of membrane protein was applied to 8%-(a) or 12%-(b) acrylamide gels for electrophoresis. M_r markers are shown as in Fig. 2; 'TD' marks the position of the tracking dye.

concentrations suggest lack of ready accessibility of the site(s) involved. Furthermore, since the M_r -26000–45000 fragment was not labelled even at 2.5 mM-[35 S]Cys-Mal in intact cells, labelling in the ghosts must have occurred either within the membrane bilayer or on the cytoplasmic membrane face. The hydrophilic nature of Cys-Mal makes it most likely that the location corresponds to that of the endofacial or Type II thiol groups described by Abbott *et al.* [40].

Fig. 6. Differential labelling and tryptic digestion of protein-depleted ghosts at a high [^{35}S]Cys-Mal concentration

The initial incubation with 50 μM -cytochalasin E (○) or -cytochalasin B (●) and unlabelled Cys-Mal was performed as described in the legend to Fig. 5, after which the ghosts were washed three times by centrifugation in 40 vol. of ice-cold phosphate buffer and labelling was carried out at 10 mM- ^{35}S]Cys-Mal (6.1 Ci/mol) for 30 min at 37 °C. The ghosts were washed twice in 120 vol. of ice-cold phosphate buffer and one aliquot from each treatment incubated without (a) or with (b) trypsin as described in the legend to Fig. 4. Data are shown from 150 μg of protein applied to 12% acrylamide gels. M_r markers are shown as in Fig. 2.

In conclusion, reaction of the exofacial carrier thiol with Cys-Mal blocks substrate translocation without affecting either substrate binding or the ability of the carrier to change orientation. These features should be useful in further studies of the role of the exofacial thiol in the transport mechanism. Additionally, the ability to label the carrier on this thiol efficiently, selectively, and to potentially a very high specific radioactivity in cells or in protein-depleted ghosts provides the means with which to identify its location in the primary carrier sequence and its relationship to sites reactive with other probes.

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