Re-examination of hexose-transporter inhibition and labelling by hexose isothiocyanates

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We have re-examined hexose-transport inhibition by hexose isothiocyanates and find that the inhibition is incomplete, probably because of decomposition of the reagent. The inhibition type is 'mixed', because hexose-transporter ligands such as maltose and cytochalasin B only give partial protection from inhibition. This suggests that a liganded-transporter-hexose isothiocyanate ternary complex is formed. We have compared the labelling of red-blood-cell membranes by [¹⁴C]MITC (D-maltose isothiocyanate) with the labelling obtained using a photoaffinity probe {ASA-BMPA [2-*N*-(4-azidosalicyloyl)-1,3-bis-(D-mannos-4'-yloxy)-2-propylamine]} which gives specific labelling of the hexose transporter in band 4.5. [¹⁴C]MITC gives a partially D-glucose-displaceable labelling of a band 3 component in the same cell preparations which show ASA-BMPA labelling of band 4.5. This eliminates the possibility that band 4.5 labelling can only occur when the HITC (hexose isothiocyanate) binding protein in band 3 is proteolysed. HITC pretreatment does not decrease ASA-BMPA labelling of the exofacial site of band 4.5. This is also consistent with the formation of ternary complex. However, HITC pretreatment inhibits both reversible and photoactivated covalent [³H]cytochalasin B binding to band 4.5. These results suggest that, in the intact cell, interactions between a band 3 HITC-binding component and the inside cytochalasin B-binding site on the hexose transporter in band 4.5 may occur.

INTRODUCTION

The use of affinity-labelling techniques to identify the protein responsible for sugar transport has produced conflicting results.

The evidence that the band 4.5 protein is the hexose transporter is extremely good. This protein can be reconstituted into vesicles which show functional hexose transport (Kasahara & Hinkle, 1977; Wheeler & Hinkle, 1981). Band 4.5 protein binds cytochalasin B, the potent hexose-transport inhibitor (Zoccoli *et al.*, 1978). Photo-labelling studies with cytochalasin B have also identified band 4.5 as the transporter protein (Carter-Su *et al.*, 1982; Shanahan, 1982). The azidosalicylate derivative of bis(mannose) (ASA-BMPA) also specifically labels the band 4.5 protein (Holman *et al.*, 1986). Furthermore, Mueckler *et al.* (1985) have recently sequenced the cDNA for this transporter protein.

On the other hand, Mullins & Langdon (1980b) conclude that band 3 is the hexose transporter. This was based on an investigation using MITC, which irreversibly inhibits hexose transport. [¹⁴C]MITC was shown to label a band 3 protein which was thought to be distinct from caproporin, the anion-transport protein. The irreversible inhibition and the labelling of the band 3 protein were both partially blocked by D-glucose and by cytochalasin B (Mullins & Langdon, 1980a,b). Mullins & Langdon (1980b) and, more recently, Kay (1985) have argued that hexose transporter migrating in the 4.5 band in SDS/polyacrylamide gels is really a breakdown product of band 3. In view of this controversy we have re-examined the possibility of a band 3 involvement in

hexose transport by using HITCs in comparison with the band 4.5 ligands, ASA-BMPA and cytochalasin B.

MATERIALS AND METHODS

Materials

ASA-BMPA was prepared as previously described (Holman & Midgley 1985; Midgley *et al.*, 1985). Radiolabel was incorporated into the 2 position of the bridge. Cytochalasin B and cytochalasin E were from Aldrich. Phloretin was from K and K laboratories. PMSF was from Sigma. D-[1-¹⁴C]Galactose, D-[U-¹⁴C]glucose, 3-*O*-[¹⁴C]methyl-D-glucose and [4-³H]cytochalasin B were from Amersham International. Other reagents and electrophoresis chemicals were from BDH or Merck.

D-Maltosylamine was synthesized from purified D-maltose (Woolfrom & Thompson, 1962) by the method of Mullins & Langdon (1980a), except that a catalytic quantity of methanol was added. D-Lactosylamine (Thomas, 1970) was synthesized in bulk by using recrystallized D-lactose in methanol saturated with NH₃.

Synthesis of MITC and LITC

These were synthesized by the method described previously (Mullins & Langdon, 1980a; Langdon, 1976). Briefly, a 50 mM-solution of the hexosylamine was prepared in 0.3 M-NaHCO₃, 30 µl of thiophosgene per ml of reaction mixture was added and the reaction mixture was stirred vigorously for 15 min. The thiophosgene that

Abbreviations used: MITC, D-maltose isothiocyanate; LITC, D-lactose isothiocyanate; HITC, hexose isothiocyanate; ASA-BMPA, 2-*N*-(4-azidosalicyloyl)-1,3-bis-(D-mannos-4'-yloxy)-2-propylamine; PMSF, phenylmethanesulphonyl fluoride.

had not reacted was then extracted twice with 5 vol. of dichloromethane. The solution was centrifuged briefly to ensure complete removal of the dichloromethane. The resulting solution was made iso-osmotic by the addition of a NaCl solution and was used immediately for reaction with cells or isolated membranes. The products of the reactions were analysed by t.l.c. using butan-1-ol/ethanol/water (13:4:2, by vol.) as solvent. These showed almost quantitative conversion into a product which was strongly absorbing at 254 nm, as described previously (Mullins & Langdon, 1980a).

Lactose is readily purified by recrystallization. D-Lactosylamine is therefore (in contrast with D-maltosylamine) readily prepared in large quantities, eliminating the need for a separate synthesis for each experiment.

Labelling and inhibition by hexose isothiocyanates

Fresh blood cells were obtained directly from volunteers. These were added directly to phosphate/saline buffer (154 mM-NaCl/12.5 mM-sodium phosphate, pH 8.0) containing PMSF (20 µg/ml) and EDTA (1 mg/ml). Cells were washed five times in phosphate/saline buffer, pH 8.0. Irreversible inhibition of hexose transport was studied by treating a 50% suspension of erythrocytes with LITC or MITC at 37 °C, with the concentrations of reagent described in the Figure legends. At the end of the incubation period the reaction was terminated by the addition of 3 ml of phosphate/saline buffer containing 100 mM-glycine. The cells were collected by centrifugation, washed again with phosphate/saline/glycine and then washed three times with phosphate/saline. The cell suspension was then adjusted to 10% haematocrit for the transport assays.

The entry of 10 µM-D-glucose or 100 µM-D-galactose was assayed at 20–22 °C. Briefly, 50 µl of a 10% cell suspension was added to 15 µl of buffer containing D-[U-¹⁴C]glucose and unlabelled D-glucose as required. After incubation the uptake was terminated by the addition of 3 ml of stopping solution composed of PBS containing 0.3 mM-phloretin and 10 µM-HgCl₂. The cells were pelleted by centrifugation and washed with a further 3 ml of stopping solution. Cell protein was precipitated by the addition of 1 ml of 10% (w/v) trichloroacetic acid and 0.5 ml of the clear supernatant was taken for counting of radioactivity. We found that the equilibrium spaces measured with D-glucose were higher than those measured with the non-metabolized sugar 3-O-methyl-D-glucose. This was especially pronounced when freshly drawn blood was used. We therefore measured the equilibrium spaces with 3-O-[¹⁴C]methyl-D-glucose, which was equilibrated with the cells for 15 min.

The rates of transport were in all cases calculated by using the integrated rate equations previously described (Eilam & Stein, 1974). Each of the reported experiments was carried out at least three times with essentially identical results. The initial rate of inactivation by HITCs was approximated from a single inactivation time of 2 min. The inactivation rate (k_{obs}) was thus calculated as:

$$k_{\text{obs}} = \ln(v_0/v)/2 \text{ min}^{-1}$$

where v and v_0 are the transport rate constants after and before inactivation respectively.

For the labelling experiments 12.5 mM-[¹⁴C]MITC (1 Ci/mol) was prepared and used as described above.

Membranes were collected and prepared for electrophoresis as described below.

Cytochalasin B binding

A 16% suspension of erythrocytes in a total volume of 400 µl were incubated with 10⁻⁸ M-[³H]cytochalasin B for 15 min at room temperature. The cells were then centrifuged for 30 s in a Microfuge and a sample of the supernatant was taken for counting radioactivity. Non-specific binding was assayed by measuring the binding of 50 µM-cytochalasin B and the non-D-glucose-displaceable binding was assayed by including 150 mM-D-glucose in the medium.

Labelling with ASA-BMPA

A 5–7 µCi portion of ASA-[2-³H]BMPA (1–2 Ci/mmol) was mixed with 800 µl of a 10% erythrocyte suspension in phosphate/saline buffer containing 320 mM-D-mannitol. These samples were placed in two 400 µl quartz thin-film demountable cuvettes (1 mm path length). The cuvettes were covered with a large 10 mm-path-length quartz cell containing 1% cumene in 2,2,4-trimethylpentane. The samples were irradiated for 15–30 s in a Rayonet RPR-100 photo-reactor (Southern New England Ultraviolet Co., Hamden, CT, U.S.A.) using RPR-3000 lamps. The cells were then washed five times in 35 ml of phosphate/saline buffer. They were then lysed at 0–4 °C in 5 mM-sodium phosphate, pH 7.8, containing 20 µg of PMSF/ml and 1 mM-EDTA, and the membranes were collected and prepared for electrophoresis.

Labelling with cytochalasin B

After pretreatment with LITC, erythrocytes were lysed and the cell membranes were collected. Membranes were resuspended at about 1 mg/ml in 5 mM-sodium phosphate, pH 7.4, in the presence of 10⁻⁴ M-cytochalasin E plus 1.6 µCi of cytochalasin B (15.5 Ci/mmol). Membranes were irradiated for 30 s in the Rayonet photochemical reactor as described above. They were then washed twice in 35 ml of 5 mM-sodium phosphate buffer, pH 7.8, and prepared for electrophoresis.

Electrophoresis

Erythrocyte membranes were mixed (buffer/membranes, 7:1, w/v) with a buffer containing 40 mM-Tris/HCl, 8% (v/v) glycerol, 8% (v/v) 2-mercaptoethanol, and 8% (w/v) SDS and were run on 5–20% (w/v) acrylamide gradient gels by using the Neville (1971) buffer system. In experiments involving [¹⁴C]MITC labelling or in a direct comparison of this label with ASA-[2-³H]BMPA labelling, the gels were sliced immediately. In other experiments involving ASA-[2-³H]BMPA labelling or [³H]cytochalasin B labelling the gels were stained with Coomassie Blue, then destained and then sliced. The slices (0.33 cm) were dried at 60 °C and then dissolved in alkaline H₂O₂ as described by Goodman & Matzura (1971). Samples were counted for radioactivity in 4 ml of scintillant of efficiencies of about 25% for ³H and 70% for ¹⁴C.

RESULTS

Transport experiments

We have found that, of a number of D-glucose isothiocyanates synthesized and tested, only those

Fig. 1. The irreversible inhibition of D-glucose uptake (●) and cytochalasin B equilibrium binding (■) by LITC

A suspension of erythrocytes was treated with 12.5 mM-LITC at 37 °C for the times indicated. The reaction was stopped by the addition of phosphate/saline/100 mM-glycine and the cells were washed and assayed for transport activity.

modified at C-1 produced extensive irreversible inhibition of glucose transport (results not shown). We found that LITC gave some advantages over MITC in its synthesis on a large scale, and we have used this compound to re-examine the inhibitory effects of HITCs on D-glucose transport. Fig. 1 shows the inhibitory effect of LITC treatment on both D-glucose transport and non-covalent cytochalasin B binding in intact erythrocytes. The loss of D-glucose-displaceable cytochalasin B binding shown closely parallels the loss of D-glucose-transport activity. Scatchard analysis of cytochalasin B binding to membranes isolated from LITC-treated cells revealed that loss of only high-affinity binding sites occurred (results not shown).

LITC and MITC consistently gave inhibition of D-glucose transport which was, however, incomplete and reached a limiting value of 50–60% inhibition. This incomplete inhibition is (at least in part) due to the instability of the reagent. T.l.c. showed substantial breakdown of the reagent after 10 min of incubation at 37 °C. If the reagent is left at 37 °C for 10 min before addition to the cells then the inhibitory effect is almost completely lost. In an attempt to test whether the residual transporters were reactive towards LITC, erythrocytes were treated with 12.5 mM-LITC for 40 min (55% inhibition), washed with PBS, and then allowed to react with freshly synthesized 12.5 mM-LITC. This resulted in some additional inhibition, but the time courses were slower, showed curvature and reached an end point of 70% inhibition when compared with untreated cells. Attempts at obtaining greater than 50–60% inhibition by using higher concentrations of reagent were inconclusive, because these higher concentrations resulted in significant cell lysis. Mullins & Langdon (1980a) reported that they were able to obtain 70% inhibition of transport, but most of their results

Fig. 2. An analysis of the effects of ligands on the saturation characteristics of the LITC reaction with the hexose transporter

Values for $k_{\text{obs.}}$ were determined by treating cell suspensions with a range of LITC concentrations in the presence or absence of ligands. After a 2 min incubation the reaction was terminated by the addition of phosphate/saline/glycine and the cells were washed six times with phosphate/saline. The calculated values of $k_{\text{obs.}}$ were then used for a secondary plot. ●, Control, $K_a = 8.9$ mM and $k_2 = 0.33$ min⁻¹; ■, plus 150 mM-D-maltose, $K_a = 13.1$ mM, $k_2 = 0.35$ min⁻¹; and ▲, plus 10 μM-cytochalasin B, $K_a = 14.3$ mM and $k_2 = 0.35$ min⁻¹. The results are the averages for three experiments for the control and two experiments in the presence of ligands. The lines were fitted by linear regression.

show only 50% inhibition. Thus it is possible that one half of the transport sites react only very slowly with HITCs, but it is not possible to test this 'half-sites' effect rigorously because of the instability and cell toxicity of the reagent.

An additional property of the HITCs not fully explored by Mullins & Langdon (1980a,b) concerns the kinetics of protection from inhibition afforded by transport-system inhibitors such as D-maltose and cytochalasin B.

The observed inactivation rate for a single-site transporter should be:

$$k_{\text{obs.}} = \frac{k_2 \cdot [\text{HITC}]}{K_a [1 + ([\text{I}]/K_i)] + [\text{HITC}]}$$

where $k_{\text{obs.}}$ is the observed inactivation rate, k_2 is the rate of conversion of an E-HITC complex to an inactive form, K_a is the affinity constant for HITC, $[\text{I}]$ is the concentration of transporter-protecting ligand and K_i the constant for its dissociation from its complex with the transporter. Thus, at concentrations of protecting ligand much higher than K_i , complete protection from inactivation should occur.

From the data in Fig. 2, K_a is calculated to be 8.9 mM and k_2 has a value of 0.33 min⁻¹. The value of K_a is similar to that previously reported for the analogous compound MITC (Mullins & Langdon, 1980a), but our

Fig. 3. A direct comparison of the labelling patterns of [^{14}C]MITC and [^3H]ASA-BMPA in freshly drawn human erythrocytes

After labelling, both batches of treated cells were processed simultaneously. (a) The distribution of radioactivity on SDS/polyacrylamide gels of membrane proteins from [^{14}C]MITC-treated cells. The erythrocyte suspension was treated with 12.5 mM-[^{14}C]MITC (1 Ci/mol) for 10 min in the presence (●) or absence (○) of 320 mM-D-glucose. Each lane carried 792 μg of membrane protein. (b) The distribution of radioactivity on SDS/polyacrylamide gels of the membrane proteins from [^3H]ASA-BMPA-treated cells. The erythrocyte suspension was photoirradiated for 30 s with 7 μCi of [^3H]ASA-BMPA in the presence (■) or absence (△) of 320 mM-D-glucose. Each lane of the gel carried 185 μg of membrane protein.

value of k_2 is much higher than the value given by those authors (0.08 min^{-1}). Those authors may have underestimated the initial rate of inactivation because of curvature in the primary plots. Even the 2 min time point we have used probably underestimates the initial inactivation rate. The observed protection from inhibition afforded by 150 mM-D-maltose and 10 μM -cytochalasin B is about 30%, and this is much less than that predicted from the dissociation constants of these compounds of 21 mM and 0.1 μM respectively. These compounds appear to be partial competitive inhibitors of the reaction with LITC, this being reflected in an increase in K_a with no effect on k_2 .

Labelling experiments

In order to explore the relationships between HITC interaction with the transporter and the known binding

sites for ASA-BMPA on the band 4.5 protein, we have directly compared ASA-BMPA and MITC labelling in a single experiment. We have also investigated the effects of HITC pretreatment on ASA-BMPA and on cytochalasin B labelling.

Fig. 3 shows the result of an experiment in which PMSF- and EDTA-treated cells were labelled either with ASA-BMPA or with MITC. The ASA-BMPA-labelled cells and MITC-labelled cells were treated simultaneously in lysing buffer containing proteinase inhibitors and were solubilized and subjected to electrophoresis at the same time. Thus any effect of proteolysis would be the same in the two halves of the experiment. The differences in labelling pattern can thus be attributed to the properties of the labels.

The most striking differences between the two labelling patterns is the extent to which the labelling is D-glucose-sensitive. The ASA-BMPA labelling is almost completely abolished by D-glucose, whereas MITC labelling is not. This must mean that most of the labelling by MITC is non-specific and nothing to do with sugar transport.

In the experiment shown there is a 27% decrease in MITC labelling in band 3 attributable to D-glucose. The average results from three experiments showed $660 \pm 163 \text{ pmol}$ of MITC bound to band 3/mg of protein. In the presence of D-glucose this was decreased to $444 \pm 97 \text{ pmol/mg}$ of protein (32% inhibition). The large amount of non-specific labelling makes identification of the protein responsible for specific (D-glucose-displaceable) labelling very difficult. The apparent M_r of the D-glucose-displaceable labelling is about 80 kDa,

Fig. 4. Distribution of radioactivity on SDS/polyacrylamide gels of membrane proteins labelled with [^3H]ASA-BMPA from (○) control erythrocytes and (●) erythrocytes pretreated with 12.5 mM-LITC at 37 °C for 10 min

After LITC treatment, transport experiments revealed a 63% decrease in the D-galactose-transport rate. Both batches of cells were photoaffinity-labelled with 7 μCi of [^3H]ASA-BMPA and the membranes collected. Each lane carried 109 μg of membrane protein.

Fig. 5. Distribution of radioactivity on SDS/polyacrylamide-gels after the photoaffinity-labelling of membranes with [3 H]cytochalasin B

Erythrocytes were pretreated with 12.5 mM-LITC for 10 min at 37 °C, and transport assays revealed that LITC treatment produced a 50% decrease in the D-galactose-transport rate. Membranes were then isolated from control and LITC-treated cells, adjusted to give equal protein concentrations, and photoaffinity-labelled with [3 H]cytochalasin B as described in the text. Each lane of the gel carried 184 μ g of protein. ○, Membranes from control cells; ●, membranes from LITC-pretreated cells.

which is distinctly different from the peak of ASA-BMPA labelling, which is about 50 kDa.

The amount of ASA-BMPA labelling is not decreased by a prior treatment with LITC (Fig. 4). However, there is an increase in labelling of the 80 kDa region. A D-galactose-transport assay was also carried out in this experiment after LITC treatment and indicated a 60% decrease in the transport rate. This is a curious result and indicates that LITC inhibits transport at a site which is separate from the ASA-BMPA-binding site. This result also supports the suggestion of ternary-complex formation in the interaction of these reagents. In the case of the labelling experiments it seems likely that the ternary complex transporter-LITC-ASA-BMPA is formed.

Although the ASA-BMPA labelling is not decreased by LITC treatment, the result of the experiment shown in Fig. 5 shows that irreversible photoactivated cytochalasin B labelling is decreased. The decrease in labelling in this case correlates well with the observed transport inhibition. It is also the result that one might expect to follow from the correlation between transport inhibition and reversible cytochalasin B binding shown in the experiment of Fig. 1.

DISCUSSION

The transporter-inhibition properties of HITCs reveal that these compounds do interact with the hexose transporter but in a more complex manner than indicated by Mullins & Langdon (1980a). Part of the complexity is due to instability of the reagent; part is due

to the observed reactivity of liganded transporter and therefore ternary-complex formation.

The labelling of membrane proteins by MITC that we have observed is consistent with the data of Mullins & Langdon (1980a,b). The calculated number of D-glucose-displaceable binding sites in band 3 is 0.7×10^5 /cell. The number of D-glucose-displaceable MITC binding sites in band 4.5 is unquantifiable or absent. The number of ASA-BMPA labelling sites in band 4.5 is 2.2×10^5 /cell (Holman *et al.*, 1986). Mullins & Langdon (1980a,b) extrapolate from the observed number of MITC binding sites to a point where 100% transport inhibition and 100% D-glucose displacement of binding might be expected. However, we have shown that neither is achievable in practice. If one does this calculation the total number of MITC binding sites is about 2.8×10^5 /cell. This is very close to the number of ASA-BMPA sites and to the number of transport sites estimated from cytochalasin B binding data. Thus this may indicate that the band 4.5 protein exists as a 1:1 molar complex with band 3 in the membrane. The association would be such that binding of HITCs to their binding site on band 3 would decrease transport via the band 4.5 protein. This effect could be mediated via a modification of the internal site of the band 4.5 protein, since cytochalasin B binding is decreased, whereas ASA-BMPA binding is not. It is clear, however, that band 4.5-band 3 interactions are not an obligatory step in transport, since band 4.5 can function alone in reconstituted systems (Wheeler & Hinkle, 1981; Baldwin *et al.*, 1982; Carruthers & Melchior, 1984).

In conclusion, the HITC effects that we have examined indicate that, in the intact erythrocyte, membrane transport through the 4.5 protein can be perturbed by interaction with a separate band 3 HITC-binding component. Re-examination of the evidence that band 3 proteolysis gives rise to hexose transporter in band 4.5 has not confirmed the postulate of Mullins & Langdon (1980b) and Kay (1985) that the work of others has produced proteolytic artefacts.

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