Exofacial photolabelling of the human erythrocyte glucose transporter with an azitrifluoroethylbenzoyl-substituted bismannose

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The synthesis of 2-*N*-[4-(1-azitrifluoroethyl)benzoyl]-1,3-bis-(*D*-mannos-4-yloxy)-2-propylamine (ATB-BMPA) is described. This compound was used as an exofacial probe for the human erythrocyte glucose-transport system. A new method is described for directly estimating the affinity for exofacial ligands which bind to the erythrocyte glucose transporter. By using this equilibrium-binding method, the K_i for ATB-BMPA was found to be $338 \pm 37 \mu\text{M}$ at 0 °C and $368 \pm 59 \mu\text{M}$ at 20 °C. This was similar to the concentration of ATB-BMPA required to half-maximally inhibit *D*-galactose uptake ($K_i = 297 \pm 53 \mu\text{M}$). The new photoaffinity reagent labelled the glucose transporter in intact cells but, because of its improved selectivity, was also used to label the glucose transporter in isolated erythrocyte membranes. The ATB-BMPA-labelled glucose transporter was 80 % immunoprecipitated by anti-(GLUT1-C-terminal peptide) antibody, which shows that the GLUT1 glucose transporter is the major isoform present in erythrocytes. The labelling of the glucose transporter at its exofacial site, and the adoption of an outward-facing conformation, renders the transport system resistant to thermolysin and trypsin treatment. Trypsin treatment of the unlabelled glucose transporter in erythrocyte membranes produced an 18 kDa fragment which was subsequently labelled by ATB-BMPA, but had low affinity for this exofacial ligand. This suggests that the trypsin-treated transporter adopts an inward-facing conformation. The ability of *D*-glucose to displace ATB-BMPA from the native transporter and from the 18 kDa trypsin fragment have been compared. The *D*-glucose concentration which was required to obtain half-maximal inhibition of ATB-BMPA labelling was 6-fold lower for the 18 kDa tryptic fragment.

INTRODUCTION

Recent studies on glucose transport in mammalian cells have shown that transport is mediated by a family of structurally similar glucose-transporter isoforms that show a tissue-specific distribution. The erythrocyte or HepG2 isoform (492 amino acids) is present in many mammalian cells [1] and is abundant in the brain [2]. There is a unique glucose-transporter isoform (509 amino acids) present in adipose and muscle tissue [3–7]. In the kidney, in pancreatic islets and in the intestine a 522-amino-acid isoform exists [8]. Foetal tissue expresses a protein which shows considerable sequence identity with the three major mammalian glucose-transporter isoforms [9]. Although each of these major isoforms occurs predominately in a particular tissue, some cross-over in distribution seems to occur. Thus adipose tissues express a small amount of the erythrocyte/HepG2 isoform in addition to the unique 509-amino-acid isoform [10,11]. The cellular abundance of these glucose transporters is not necessarily balanced by their availability at the cell surface in a form which can transport sugars. Thus techniques which can mark the glucose transporter at the cell surface (such as an affinity label) can be used in conjunction with specific antibodies to quantify the proportion of each isoform exposed at the membrane.

To meet the requirement for a probe which can be used to quantify glucose-transporter levels at the cell surface, we have developed a series of bis-hexoses [12,13]. We have found that 1,3-bis-(*D*-mannos-4-yloxy)-2-propylamine (BMPA) is particularly useful. This has an amino group between the *D*-mannose moieties that can be coupled to a range of photoactivatable groups. The resulting compounds are hydrophilic and impermeable. The derivatives have higher affinity for the glucose-transport system than the parent compound, *D*-mannose, but have much lower affinity than alternative labelling reagents such as cytochalasin B

[14,15] and IAPS-forskolin [16]. Thus it is important that the photolabile substituent carried by the bismannose is selective, otherwise non-specific interactions with other proteins predominate in the labelling patterns observed on gels. We have found that nitrophenyl azides are not very selective. The azidosalicyl derivative of BMPA (ASA-BMPA) is much better and has allowed selective labelling of the human erythrocyte glucose transporter [17]. Part of the selectivity of this compound is, however, due to its impermeability; its access to non-specific labelling sites at the inner surface of the membrane is thus abolished. When used with isolated membranes, ASA-BMPA gave some labelling of erythrocyte membrane band 3 and spectrin. The carbene precursor benzophenone derivative of BMPA [18] is much more selective than the phenylazide derivatives, but requires relatively long irradiation times to photolabel the glucose transporter. An alternative is to use a diazirine substituent. The general use of diazirines as carbene precursors and as photolabels has been discussed by Bayley & Knowles [19]. We have previously described the use of diazirine substituted hexoses as transported substrates for the glucose-transport system [20]. The lipid-soluble compound diazirine 3-trifluoromethyl-3-(*m*-iodophenyl)diazirine [21] has been used to study the hydrophobic regions of membrane proteins.

The synthesis of a new photolabel, 2-*N*-[4-(1-azitrifluoroethyl)benzoyl]-1,3-bis-(*D*-mannos-4-yloxy)-2-propylamine (ATB-BMPA), is described here, together with its erythrocyte-glucose-transporter-labelling properties.

MATERIALS AND METHODS

Preparation of ATB-[2-³H]BMPA

A 12 mg portion of 4-(1-azitrifluoroethyl)benzoic acid (Bachem) plus 14 mg of hydroxybenzotriazole were dissolved in

Abbreviations used: ASA-BMPA, 2-*N*-(4-azidosalicyl)-1,3-bis-(*D*-mannos-4-yloxy)-2-propylamine; ATB-BMPA, 2-*N*-[4-(1-azitrifluoroethyl)benzoyl]-1,3-bis-(*D*-mannos-4-yloxy)-2-propylamine; GLUT1, erythrocyte/brain glucose-transporter isoform; IAPS, 3-iodo-4-azidophenethylamido-7-*O*-succinyldeacetyl.

450 μ l of dimethylethylene glycol in a Microfuge tube; 20 mg of dicyclohexylcarbodiimide in 150 μ l of dimethylethylene glycol was then added and the reaction was allowed to proceed at 4 °C overnight. The tube was centrifuged briefly to pellet the dicyclohexylurea, and 400 μ l of the clear supernatant was added dropwise to a solution containing 25 mCi of [2-³H]BMPA in 50 μ l of methanol and 3 μ l of *N*-methylmorpholine as base. After 2 h at room temperature, t.l.c. analysis in ethyl acetate/methanol/water (9:2:1, by vol.) showed approx. 70% conversion into ATB-[2-³H]BMPA. The product was separated from minor radiolabelled impurities by paper chromatography in butanol/ethanol/water (49:11:19, by vol.). The yield was 16 mCi and the specific radioactivity was 10 Ci/mmol. Complete removal of hydroxybenzotriazole was not achieved, since this compound has only slightly higher mobility than ATB-BMPA.

Preparation of ATB-BMPA

The poor separation of ATB-BMPA from hydroxybenzotriazole referred to above meant that an alternative method had to be used for the preparation of non-labelled ATB-BMPA. A 5 μ l portion of isobutyl chloroformate was added to 12 mg of 4-(1-azi-2,2,2-trifluoroethyl)benzoic acid in 50 μ l of dimethylformamide and 5 μ l of *N*-methylmorpholine at 0–4 °C. A 25 mg portion of BMPA was dissolved in 200 μ l of dimethylformamide and 5 μ l of *N*-methylmorpholine. The two solutions were then mixed. After 2 h at 0–4 °C, the solvents were removed and the product was applied to a 5 ml silica-gel column which was eluted with ethyl acetate/methanol/water (18:2:1, by vol.). Combined fractions containing the product were rechromatographed to give pure product. The yield was 15 mg. The mass spectrum (negative-ion fast-atom bombardment) gave $m/z = 626$ [M^+ (the molecular ion)–1]. Chromatography of radiolabelled and unlabelled samples showed that radioactivity, u.v. absorbance and sugar-staining material co-chromatographed.

Estimation of the affinity constant for ATB-BMPA

The affinity constant (or half-maximal inhibition constant) was determined either by the inhibition of the uptake of 100 μ M-D-galactose or by determining the inhibition of the reversible binding of the exofacial ligand ASA-BMPA. For transport-inhibition determinations, 50 μ l of an erythrocyte suspension (40% cytocrit) was mixed with 10 μ l of phosphate/saline buffer (see below) containing the indicated concentrations of ATB-BMPA and 10 μ l of D-[1-¹⁴C]galactose (100 μ M final concn.). Transport was stopped after 10 s by the addition of a stopping solution containing 0.3 mM-phloretin in phosphate/saline buffer. Uptake rates were then calculated as described in [12].

For the binding experiments, 200 μ l of erythrocyte suspensions at high cytocrit (> 80%) were mixed with 10 μ l of buffer containing 0.1 μ Ci (10 pmol) of ASA-[2-³H]BMPA and 0.02 μ Ci of [U-¹⁴C]sucrose and non-labelled ATB-BMPA at a range of dilutions. Samples were mixed and incubated for 5 min and then spun in a Microfuge. These operations (including centrifugation) were carried out either at 20 °C or at 0–4 °C. A 5 μ l sample of the supernatant was then taken. Extracellular space and the final total concentrations of ATB-BMPA were then calculated from the [¹⁴C]sucrose in this sample. Bound/free ASA-BMPA was calculated as $[(H_{mix}/C_{mix})/(H_{spl}/C_{spl})]-1$. Bound ASA-BMPA was calculated as $1-[(H_{spl}/C_{spl})/(H_{mix}/C_{mix})]\cdot Tot$ (where H_{mix} is ³H d.p.m. in the isotope mixture without cells, C_{mix} is the ¹⁴C in the isotope mixture, H_{spl} is the ³H in the sample equilibrated with the cells, C_{spl} is the ¹⁴C in the sample and Tot is the added concentration of ligand). In additional samples 50 μ M-cytochalasin B was added to estimate the non-specifically bound ASA-BMPA (~ 10% of the total). The small bound/free ratio in the presence of cytochalasin B was subtracted from that

obtained in its absence. At 80% cytocrit a typical bound/free ratio for ASA-BMPA was 0.47, which is consistent with an affinity constant for ASA-BMPA of ~ 60–80 μ M [17] and a concentration of binding sites in a 80% suspension of erythrocytes of ~ 35 μ M in the extracellular space (on the basis of 7 μ mol/litre of packed cells [22,23]). This assay provides a quick and convenient estimate of affinity constants and is easier to perform than that based on inhibition of substrate-transport rates.

Photolabelling of erythrocytes and erythrocyte membranes

Erythrocytes from 1–3-week-old transfusion blood were washed five times in phosphate/saline buffer (154 mM-NaCl/12.5 mM-sodium phosphate, pH = 7.2). A 200 μ l portion of erythrocyte suspension (20% cytocrit) was mixed with 10–30 μ Ci of ATB-[2-³H]BMPA in 200 μ l of solution (usually containing D-mannitol or D-glucose) in 1-mm-pathlength cuvettes. The cuvettes were covered with a 10-mm-pathlength quartz cell containing a chemical filter of 1% cumene in 2,2,4-trimethylpentane. Samples were irradiated for 60 s in a Rayonet RPR-100 photoreactor containing RPR-3000 lamps. After irradiation, the cells were washed five times in phosphate/saline buffer. The membranes were then obtained by hypo-osmotic lysis [17].

Photolabelling of erythrocyte membranes with ATB-BMPA and with cytochalasin B was carried out using the methods previously described [17,24]. Erythrocyte membrane suspensions at 300 μ g of membrane protein/300 μ l in 5 mM-sodium phosphate buffer, pH 7.2, were mixed with 1.6 μ Ci of [4-³H]cytochalasin B in the presence of 10⁻⁴ M-cytochalasin E or with 10 μ Ci of ATB-BMPA and were irradiated for 30–60 s in 1-mm-pathlength cuvettes. After irradiation, membranes were diluted with 2.5 ml of phosphate buffer and centrifuged at 20000 *g* for 20 min. The pellets were resuspended and washed twice more in phosphate buffer to remove non-bound radiolabel.

Enzyme treatments

Erythrocyte membrane suspensions, 1 mg/ml in 5 mM-sodium phosphate buffer, pH 7.2, were prepared as described in [17,24]. These suspensions were treated either with trypsin at 100 units/ml for 30 min at 20 °C or with thermolysin at 0.4 unit/ml for 30 min at 20 °C. After treatment with trypsin, the enzyme was removed by dilution of the membranes in 3.0 ml of 5 mM-phosphate buffer, pH 7.2, and by centrifugation at 20000 *g* for 20 min. The pellet was resuspended and washed again in the same buffer. After thermolysin treatment the enzyme was removed by dilution of the membranes in 3.0 ml of 5 mM-phosphate buffer with 27 mM-EDTA (pH 7.2) and by centrifugation at 20000 *g* for 20 min. After washing of trypsin- and thermolysin-treated samples, the membranes were resuspended in phosphate buffer without enzymes.

Immunoprecipitation

The antibody used was raised against a synthetic 15-amino-acid peptide (CGEELFHPLGADSQV) corresponding to the C-terminal 13-amino-acid sequence reported by Mueckler *et al.* [1] and with a glycine spacer before the cysteine used in coupling. The methods used were those described by Davies *et al.* [25]. The crude antiserum was affinity-purified by using alkali-treated erythrocyte membranes [26]. A 100 μ g portion of photolabelled erythrocyte membranes were solubilized in 300 μ l of a solution containing 0.5% Mega 10 [27], 5 mM-sodium phosphate buffer and 2 mM-dithiothreitol, pH 7.2. These samples were then treated with 135 μ g of affinity-purified C-terminal antibody and 30 μ l of Protein A-Sepharose. The samples were mixed for 1–2 h at 0–4 °C. The Protein A-antibody complex was sedimented by

centrifugation, and then the supernatant was removed and re-treated to obtain a second precipitate. The pellets were washed by resuspension in $2 \times 50 \mu\text{l}$ of 0.5% Mega 10 solution (as described above) and the washings were combined with the first supernatant. To the combined supernatant sample was added $150 \mu\text{l}$ of solubilizing buffer containing 10% (w/v) SDS and 36% (v/v) glycerol, with 10% mercaptoethanol. Protein was released from the antibody pellets (usually the combined first and second pellets) with $200 \mu\text{l}$ of the same solubilizing buffer. Both the supernatant and the pellet samples were then subjected to electrophoresis. The positions of the photolabelled peaks were compared with those of the molecular-mass-marker proteins (BSA, 66 kDa; ovalbumin, 45 kDa; soya-bean trypsin inhibitor, 20 kDa; lysozyme, 14 kDa), as indicated in the Figures.

Electrophoresis

Samples in solubilizing buffer (see above) were run on 16 cm 10% (or 12%) polyacrylamide gels using the Laemmli [28] discontinuous buffer system. The gels were stained briefly with Coomassie Blue, then destained and sliced (three slices/cm). The slices (in scintillation vials) were dried at 80°C for 2–3 h, then dissolved in $300 \mu\text{l}$ of H_2O_2 [containing 2% (v/v) NH_3 solution] for a further 2–3 h at 80°C . Liquid-scintillation fluid was then added and the radioactivity was counted.

RESULTS

Structure and photodecomposition of ATB-BMPA

The structure of ATB-BMPA is shown in Fig. 1. It contains two D-mannose groups linked through their C-4 hydroxy groups to 2-propylamine. The photolabile 4-(1-azido-2,2,2-trifluoroethyl)-benzoic acid group [29] is coupled to the amino group on the bridge and between the two D-mannose groups. In this position the bulky group does not interfere with the interaction of the transporter with the hexose hydroxy groups, which are required for binding [30].

By using our apparatus, the half-time of photolysis of ATB-BMPA in water is approx. 30 s (Fig. 2). This is considerably longer than the half-times observed with phenylazides [17] but, as shown below, the resulting carbene is more selective than the nitrenes derived from the activation of phenylazides.

Determination of the affinity constant for ATB-BMPA

We have used two methods to determine the affinity (or half-maximal inhibition constant, K_i) of the erythrocyte glucose-transport system for ATB-BMPA. Fig. 3 shows the inhibition of $100 \mu\text{M}$ -D-galactose uptake at 20°C . The calculated K_i was $297 \pm 53 \mu\text{M}$. The new method for determining the same parameter is based on inhibition of the equilibrium binding of ASA-BMPA. The displacement of radiolabelled ASA-BMPA by non-

Fig. 1. Structure of ATB-BMPA

Fig. 2. Photochemical decomposition of ATB-BMPA

ATB-BMPA ($150 \mu\text{M}$ in water) was irradiated in a Rayonet photochemical reactor with 300 nm lamps for 0, 15, 30 and 45 s as indicated. The absorption maximum at 350 nm gave an ϵ value of $380 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

labelled ATB-BMPA gave a K_i of $368 \pm 59 \mu\text{M}$ at 20°C . The equilibrium binding was also studied at 0 – 4°C , but no change in the affinity constant was observed ($K_i = 338 \pm 37 \mu\text{M}$ at 0 – 4°C) (Fig. 4).

ATB-BMPA photolabelling

When ATB-BMPA was irradiated in the presence of intact erythrocytes, good incorporation of radiolabel into the glucose-transport system occurred. No other membrane proteins were labelled, and the photolabel was completely displaced from the glucose transporter by high concentrations of cytochalasin B and by D-glucose (Fig. 5a). Fig. 5(b) shows that the C-terminal antibody that we have prepared precipitated approx. 64% of the glucose transporter after a single treatment. A second treatment (pellet 2 in the Figure), gave a further 20% (56% of the first supernatant). Thus the combined treatments gave over 80% precipitation. Photolabelled transporter was not precipitated by preimmune serum (results not shown).

Fig. 5(c) shows that the new photolabel, ATB-BMPA, is

Fig. 3. Inhibition of D-galactose uptake by ATB-BMPA

The erythrocyte uptake of $100 \mu\text{M}$ -D-galactose was measured at a range of ATB-BMPA concentrations. The reciprocal of the uptake rate constant (s/v) was plotted against the ATB-BMPA concentration. The K_i was determined by non-linear regression fitting to the Michaelis-Menten equation. The K_i was $297 \pm 53 \mu\text{M}$ (from two experiments with triplicate observations at each inhibitor concentration); results are means and the bars represent the S.E.M. of the six observations at each concentration.

Fig. 4. Inhibition of ASA-BMPP binding by ATB-BMPP

The binding of 0.1 μCi (10 pmol) of ASA-BMPP to 200 μl of a 80% cytochrome erythrocyte suspension was measured over a range of ATB-BMPP concentrations. The free/bound ratio was calculated by comparison with [^{14}C]sucrose as described in the Materials and methods section. The free/bound ratio in the presence of ATB-BMPP was multiplied by the bound/free ratio in the absence of inhibitor (n) and was plotted against the ATB-BMPP concentration. The K_i was calculated by non-linear regression fitting to the Michaelis-Menten equation. At 20 $^{\circ}\text{C}$ ($\circ$) the $K_i = 368 \pm 59 \mu\text{M}$ (from observations at 16 ATB-BMPP concentrations). At 0 $^{\circ}\text{C}$ ($\bullet$) the $K_i = 338 \pm 37 \mu\text{M}$ (from observations at 14 ATB-BMPP concentrations).

sufficiently selective to be used with isolated erythrocyte membranes. The labelling of the glucose transporter in isolated membranes is very efficient, and large amounts of radioactivity are incorporated: 90 pmol of transporter (calculated by assuming 600 pmol/mg of membrane protein) incorporated 5.7 pmol of ATB-BMPP, and therefore 6.3% of the sites are labelled. The concentration of ATB-BMPP in the reaction mixture was 20 μM , which means that, as the half-saturation constant is 350 μM , the sites were 5.4% saturated. The efficiency of labelling was therefore calculated as 117%. Another experiment showed labelling to be 177% efficient. These values seem higher than expected, but suggest that if all the sites were occupied by ligand, then all the sites would covalently cross-link with the ATB-BMPP.

Enzyme treatments

Fig. 6(a) shows trypsin treatment of membranes isolated from ATB-BMPP-labelled cells. The labelled glucose transporter was converted into an 18 kDa fragment, but a large amount of a labelled 50 kDa transporter remained. In the same experiment we treated isolated membranes with trypsin and then labelled them with ATB-BMPP subsequently (Fig. 6b). There was much less conversion into the 18 kDa peak when the glucose transporter was labelled first.

Fig. 6(a) also shows that, after trypsin treatment, the ATB-BMPP-labelled glucose transporter, which ran in the 50 kDa region of the gel, and the 18 kDa trypsin fragment, have lost a ~ 4 kDa C-terminal peptide, as they were not immunoprecipitated by the anti-(C-terminal peptide) antibody.

We also studied the proteolysis of the glucose transporter by

Fig. 5. Photolabelling of the glucose transporter

(a) ATB-BMPP photolabelling of the glucose transporter in intact erythrocytes. The labelling by ATB-[2- ^3H]BMPP was compared in the absence of D-glucose ($\bullet$) and at 1 mM- ($\circ$), 5 mM- ($\triangle$), 25 mM- ($\blacksquare$) and 100 mM- ($\blacktriangle$) D-glucose. Binding and labelling was completely displaced by 50 μM -cytochalasin B ($\square$). Membranes were prepared and the labelled proteins were analysed by electrophoresis. Only the glucose-transporter region of the electrophoresis gel is shown for clarity. There were no other labelled peaks in other regions of the gel. (b) Immunoprecipitation of ATB-BMPP-labelled glucose transporter. Glucose transporter was labelled in intact erythrocytes by ATB-[2- ^3H]BMPP and then membrane suspensions were prepared. Glucose transporter was then immunoprecipitated with anti-(C-terminal peptide) antibody as described in the Materials and methods section. The radioactivity was mainly associated with the first pellet ($\bullet$). The supernatant when subjected to further treatment with antibody gave a second pellet ($\circ$), but some transporter remained in the supernatant ($\blacktriangle$). These samples were analysed by electrophoresis. (c) ATB-BMPP photolabelling of the glucose transporter in isolated erythrocyte membranes. Membrane suspensions in phosphate buffer were labelled with ATB-[2- ^3H]BMPP in the presence of 300 mM-D-mannitol ($\bullet$) or 300 mM-D-glucose ($\circ$). The membranes were then washed in phosphate buffer and analysed by electrophoresis. In this and succeeding Figures the arrowheads ($\blacktriangledown$) indicate the positions of marker proteins (see the text).

Fig. 6. Trypsin treatment of the glucose transporter

(a) Trypsin treatment of ATB-BMPA-labelled glucose transporter. The glucose transporter was photolabelled with ATB-[2- ^3H]BMPA in intact erythrocytes. Membranes were then prepared and resuspended in phosphate buffer. The membrane suspensions were then treated with 100 units of trypsin/ml for 30 min at 20 °C. The trypsin was removed by washing in phosphate buffer. The labelled trypsin-treated membranes were then subjected to immunoprecipitation with anti-(C-terminal peptide) antibody to give a supernatant sample (●) and a pellet (○) which were analysed by electrophoresis. (b) Photolabelling of the glucose transporter after trypsin treatment. Erythrocyte membranes were treated with 100 units of trypsin/ml for 30 min at 20 °C. The membranes were washed in phosphate buffer to remove the trypsin and were then photolabelled with ATB-[2- ^3H]BMPA. The membranes were then

thermolysin. The ATB-BMPA-labelled transporter was much less susceptible to cleavage by this enzyme than the cytochalasin B-labelled transporter. Fig. 7(a) shows that thermolysin at 0.4 unit/ml completely cleaved the cytochalasin B-labelled transporter into an 18 kDa fragment. Fig. 7(b) shows that a concentration of thermolysin six times higher than this (2.4 units/ml) gave very little proteolysis of the ATB-BMPA-labelled transporter. Fig. 7(b) also shows that, after thermolysin treatment, the ATB-BMPA-labelled glucose transporter was fully precipitated by the antibody, which suggests that the C-terminus was still present.

Examination of D-glucose displacement of ATB-BMPA from the glucose transporter

We compared the labelling of the glucose transporter in isolated erythrocyte membranes before (Fig. 5c) and after (Fig. 6b) trypsin treatment. Trypsin-treated membranes showed 10–12-fold less labelling (two experiments), which suggests that the affinity for ATB-BMPA is markedly reduced in the trypsin fragment. We have also compared the effect of varying the D-glucose concentration on the ATB-BMPA labelling of the native glucose transporter and the transporter in the trypsin fragment. As described above, the binding of ATB-BMPA to the native glucose transporter was inhibited by D-glucose and by cytochalasin B. Fig. 5(a) shows that the native transporter required a high D-glucose concentration to displace all the ATB-BMPA photolabelling, and even 100 mM-D-glucose showed some residual labelling, which was only completely displaced by cytochalasin B. In contrast with the result obtained with the native glucose transporter, Fig. 8(a) shows that 2 mM- and 10 mM-D-glucose gave virtually complete displacement of the ATB-BMPA photolabel from the trypsin fragment, and the residual labelling was similar to that obtained with 50 μM -cytochalasin B.

The concentration of D-glucose which gave half-maximal displacement from the native transporter was about 10 mM. As shown in Fig. 8(b), this could be due to D-glucose combining with two sites (curve 2) or to a single site (curve 3). Fig. 8(b) also shows that, after trypsin treatment, the D-glucose affinity constant is markedly reduced and is around 1.5 mM (curve 1).

DISCUSSION

Photoaffinity labelling with bis-hexoses

We have previously shown that the aryl azide derivative of bis-D-mannose, ASA-BMPA, can be successfully used for photolabelling the hexose transporter of human erythrocytes [17]. We have used this compound to probe the location of the exofacial hexose-binding site in the transporter [24]. Labelling at the external and internal sites occurs in the highly conserved helices 9, 10 and 11 [24,31]. As reported [18], ASA-BMPA and many additional aryl azide derivatives of bismannose and bisglucose failed to give selective labelling of glucose transporters where these are not very abundant in the cell membrane. Thus we have sought to develop more selective bis-hexose photolabels. The new compound, ATB-BMPA, is shown to be easily synthesized from BMPA and the (azitrifluoroethyl)benzoic acid developed by Nassal [29] that can now be obtained commercially. We have shown that radiolabelled ATB-BMPA required for

washed in phosphate buffer and analysed by electrophoresis. Photolabelling was compared in the presence of 300 mM-D-mannitol (●) and 300 mM-D-glucose (○). In the same experiment ATB-BMPA was used to label membranes which were not trypsin-treated. This showed 12-fold higher glucose-transporter labelling (Fig. 5c).

Fig. 7. Thermolysin treatment of the glucose transporter

(a) Thermolysin treatment of cytochalasin B-labelled glucose transporter. The glucose transporter in erythrocyte membranes was labelled with cytochalasin B. The membranes were then washed and resuspended in phosphate buffer. The membrane suspension was then treated with 0.4 unit of thermolysin/ml for 30 min at 20 °C. The membranes were then washed and resuspended in phosphate buffer with EDTA. They were then subjected to electrophoresis, and the labelled glucose-transporter fragments (●) were analysed. (b) Thermolysin treatment of ATB-BMPA-labelled glucose transporter. The glucose transporter was labelled with ATB-[2-³H]BMPA in intact erythrocytes and then the membranes were isolated. The membrane suspension in phosphate buffer was then treated with 2.4 units of thermolysin/ml for 30 min at 20 °C. The membranes were washed in phosphate buffer with EDTA. Half of the sample was then subjected to immunoprecipitation with anti-(C-terminal peptide) antibody. This gave a pellet (○) and a supernatant (▲). The labelled proteins in these samples were analysed by electrophoresis and were compared directly with the half of the sample which was not immunoprecipitated (●).

Fig. 8. Ligand binding to the glucose transporter

(a) Glucose displacement of ATB-BMPA from trypsin-treated glucose transporter. Erythrocyte membranes were treated with trypsin (100 units/ml) for 30 min at 20 °C. The enzyme was removed by washing the membranes with phosphate buffer. Membrane suspensions were then labelled with ATB-[2-³H]BMPA, either without D-glucose (●) or with D-glucose at 2 mM (▲) and 10 mM (○). The membranes were then washed in phosphate buffer and analysed by electrophoresis. The trypsin fragment in the 18 kDa region of the gel is shown. (b) A comparison of D-glucose displacement of ATB-BMPA from the native transporter and from the trypsin fragment. The ratio of the areas of the photolabelled peaks obtained in the presence and absence of D-glucose (A/A_0) was plotted against the D-glucose concentration. The ATB-BMPA labelling that was not displaced by 50 μ M-cytochalasin B was subtracted. Displacement by D-glucose of ATB-BMPA labelling of the intact transporter (■) gave a K_1 of 10 mM when results were fitted to the Michaelis-Menten equation. Displacement of ATB-BMPA from the trypsin fragment (▽) showed a K_1 of about 1.5 mM [curve 1, $A/A_0 = 1.5/(1.5 + G)$, where G is the D-glucose concentration]. Curve 2 was obtained from the equation for a two-site model [36]:

$$A/A_0 = \frac{1}{2} [1.5/(1.5 + G)] + \frac{1}{2} [26/(26 + G)]$$

Curve 3 was obtained from the single-site model [$A/A_0 = 10/(10 + G)$].

photolabelling can be synthesized in good yield from radiolabelled BMPA. The BMPA is most effectively obtained by catalytic reduction of a bis-(D-mannose) oxime [18].

The results show that the new reagent can be used as a marker

for cell-surface-exposed glucose transporters and that this probe is sufficiently selective to be used in isolated erythrocyte membrane preparations. Immunoprecipitation of photolabelled glucose transporters with anti-(GLUT1 C-terminal peptide) antibody has shown that the GLUT1 isoform is the major isoform in erythrocytes.

Proteolysis of photolabelled glucose transporters

The new photolabel and the anti-(C-terminal peptide) antibody have been used together to examine the effects of trypsin and thermolysin proteolysis of the erythrocyte glucose transporter. We have shown that, when erythrocyte membranes are treated with trypsin first, and then subsequently labelled with ATB-BMPA, very extensive conversion into the 18 kDa fragment occurs. In contrast with this, trypsin treatment of the ATB-BMPA-labelled glucose transporter has shown that bound ATB-BMPA reduces the rate of trypsin reaction, probably in the central loop region of the transporter. The first site to be cleaved is at the C-terminus, which leaves a labelled 50 kDa transporter which is not immunoprecipitated by anti-(C-terminal peptide) antibody. A slower second cleavage occurs to give the 18 kDa fragment. The slowing of the trypsin reaction suggests that glucose transporter with an exofacial label covalently attached is relatively resistant to the trypsin, because of the adoption of an outward-facing conformation. These findings support those of Davies *et al.* [25], who analysed the two sites of trypsin cleavage that result in the 18 kDa glucose transporter fragment, and of Gibbs *et al.* [32], who showed that 4,6-*O*-ethylidene-D-glucose (which binds reversibly to the outside site) reduced the rate of trypsin cleavage.

We have also shown here that thermolysin cleaves ATB-BMPA-labelled glucose transporters much less rapidly than it cleaves cytochalasin B-labelled transporters. This is consistent with our previous comparison of ASA-BMPA-labelled and cytochalasin B-labelled glucose transporters [24], but we have now shown that the ATB-BMPA-labelled glucose transporter is resistant to thermolysin cleavage at both the central region and the C-terminus, since the transporters are immunoprecipitated after labelling and thermolysin treatment.

There appears to be an increase in susceptibility to proteolysis in the order: exofacial conformation < unliganded conformation(s) < endofacial conformation. The measurement of these changes that have been described here and elsewhere [24,32,33] may be an important approach for studying transporter structural changes that underlie the transport kinetics. The combination of enzyme treatment with the use of affinity labels and antibodies directed against specific regions of the transporter may be particularly useful as an approach to explore further the structure-function link.

Affinity constants for glucose-transporter ligands

The affinity or half-maximal inhibition constants for ATB-BMPA obtained from transport inhibition and from binding experiments are similar and are $\sim 300 \mu\text{M}$ at 20 °C. The affinity constant for ATB-BMPA inhibition of ASA-BMPA binding is unaffected by cooling the samples to 0–4 °C. At this temperature the K_i is also $\sim 300 \mu\text{M}$. Thus there is a lack of any marked temperature-dependence for this exofacial ligand. We have previously shown that the K_i for nitroazidophenyl-BMPA inhibition of D-galactose transport in erythrocytes is around $300 \mu\text{M}$ at 0, 20 and 37 °C [12]. Other simpler exofacial ligands, such as D-maltose and 4,6-*O*-ethylidene-D-glucose, also show K_i values which are not very temperature-sensitive (G. D. Holman, unpublished work), so this is a general effect that occurs with sugars having various hydrophilicities. These are perhaps surprising findings which are not immediately reconcilable with the

proposed redistribution of carriers that is thought to occur over this range of temperatures [34]. The K_i is dependent on the equilibrium dissociation constant:

$$K_i = K_{\text{diss.}} \cdot (1 + A) \quad (1)$$

(where A is the asymmetry in the unloaded carrier distribution; that is, the rate constant for empty carrier movement from outside to inside over the rate constant for empty carrier movement from inside to outside). Thus a lowering of temperature, which increases A , would have to produce a corresponding decrease in $K_{\text{diss.}}$ so that there is no overall effect on the K_i . In present formulations of carrier kinetics these constants (A and $K_{\text{diss.}}$) are unrelated.

There is a marked change in the affinity for ATB-BMPA and for D-glucose after trypsin treatment. The reduction in ATB-BMPA labelling to the 18 kDa trypsin fragment suggests that the affinity for this outside ligand is markedly reduced (~ 12 -fold). In contrast with this the D-glucose affinity is increased ~ 6 -fold. We have previously shown that cytochalasin B photolabelling of the trypsin fragment is only slightly less effective than that obtained with the unproteolysed glucose transporter [33]. Measurements of equilibrium binding of cytochalasin B to the glucose transporter in trypsin-treated erythrocyte membranes have shown the same effect [31]. Cairns *et al.* [31] have shown that lower concentrations of D-glucose were required to reduce cytochalasin B binding to the trypsin-treated membranes than were required to reduce cytochalasin B equilibrium binding to the unproteolysed glucose transporter. Together with our new findings these observations indicate that the glucose transporter has adopted an inward-facing conformation after proteolysis. We were also led to this conclusion by the results of Dr Y. Oka (personal communication). Dr Oka has discovered that a C-terminal deletion mutant of the HepG2 type of glucose transporter, when transfected into Chinese-hamster ovary cells, has an inside-directed conformation. The clones containing the deletion mutant show very poor binding of ATB-BMPA, but good binding of cytochalasin B. This inside-directed conformation is associated with the loss of sugar transport that is shown by these clones. The transporter is thus very similar to the trypsin fragment of the erythrocyte transporter, which also shows loss of glucose transport [35].

Eqn. (1) shows that a 10-fold increase in A (the asymmetry in free carrier distribution) and the adoption of an inward-facing conformation can account for the loss of affinity for ATB-BMPA at the exofacial site. However, the K_i for D-glucose displacement of a ligand is given by:

$$K_i = K_{\text{diss.}} \cdot (1 + A)/(1 + B) \quad (2)$$

(where B is the inside/outside-loaded carrier-distribution ratio). Thus the increase in B has to be much larger (~ 60 -fold) than the increase in A (~ 10 -fold) to account for the lowering of the D-glucose K_i that we have observed. The possibility of allosteric interactions [36–39] has not been completely eliminated and could also account for the observed D-glucose displacement. However, the fact that such a lot of the transport data now available can be accommodated by the simple carrier model [34,40,41] is impressive.

Labelling the glucose transporter in other tissues

The human erythrocyte glucose-transporter isoform GLUT1 is present in many cell types [1], and in many cases occurs with other transporter isoforms. Thus the technique of exofacial labelling combined with the use of isoform-specific anti-(C-terminal peptide) antibodies that we have described will be useful in identifying and quantifying the proportion of each isoform

exposed at the cell surface in other tissues (such as insulin-responsive tissues). Comparisons with studies using other labeling reagents, such as cytochalasin B and IAPS-forskolin [16] and thiol reagents [42] should provide information on the accessibility of internal, external and modifier sites in these glucose-transporter isoforms.

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