Biochimica et Biophysica Acta 812 (1985) 503–511
Elsevier

BBA 72397

Characterization of a photosensitive glucose derivative. A photoaffinity reagent for the erythrocyte hexose transporter

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(Received September 26th, 1984)

Key words Photoaffinity probe; Glucose derivative; Transport regulation; Hexose transporter, (Erythrocyte)

The photosensitive reagent 6-*N*-(4-azido-2-hydroxy-3,5-diiodobenzoyl)-D-glucosamine has been assessed as a potential photoaffinity label for the hexose transporter. Under zero-trans conditions, transport experiments performed in the dark reveal that the reagent inhibits the uptake of D-glucose in resealed human erythrocyte ghosts. Increasing the concentration of glucose in the transport medium has a protective effect, reducing the inhibition. Kinetic analysis indicates that the probe acts as a competitive inhibitor with high affinity for the erythrocyte hexose transporter (K_i between 0.07 and 0.2 μM). Exposure to a 280 nm filtered high intensity mercury-vapor lamp results in a rapid and efficient photolysis. At low concentrations of the probe, specific labeling of membrane preparations was observed. Autoradiograms of 10% SDS gels revealed the specific labeling of bands 4.51 and 6. This labeling was concentration-dependent and protected by D-glucose (not the L-isomer) and phloretin in the medium. When subjected to multiple exposures of low concentration of the photoaffinity reagent, apparent saturation was achieved.

Introduction

Photoaffinity probes have emerged as a powerful class of affinity reagents enabling highly specific labeling and identification of proteins in complex mixtures. To facilitate the study of the glucose transport process, it was desirable to design a photoaffinity label which could be recognized by the hexose transporter. In order to be effective, the photoaffinity probe has to be an efficient light absorber, have high affinity and specificity, limited nonspecificity and be radiolabeled with high specific activity to enable detection by autoradiographic analysis [1]. A probe of this type would be useful in the identification of transport protein(s) as well as in revealing changes in the number of transporters and identifying functionally available sites.

Our approach in developing a hexose transport photolabel was to incorporate the natural substrate glucose, but render it somewhat hydrophobic in the hopes of increasing its affinity for the transporter. Hydrophobic character was postulated to be important in imparting high affinity when it was realized that most of the transport protein inhibitors which have affinities 3 orders of magnitude greater than that of glucose for the transporter are highly hydrophobic; in addition, much of the data on the transport protein supported the hypothesis that it was an integral membrane protein.

Several photoaffinity reagents were developed in our laboratory [2] attaching a glucose molecule to an azide-modified fatty acid chain. Most of

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Abbreviation: AHDB-GlcN, 6-*N*-(4-azido-2-hydroxy-3,5-diiodobenzoyl)-D-glucosamine.

these labels were competitive; however, they were also very insoluble in water and proved to be lysogenic. More recently, D-galactose and D-glucose aryl azide derivatives have been synthesized [3]. Fig. 1 shows the structure of the C-6-substituted glucose probe used in this study These reagents are advantageous in that the aryl azide lends a greater chemical stability to the probe than other azides, and also imparts a significant hydrophobic character to the molecule. In addition, unlike other generally used aryl azides, an hydroxyl was substituted for the nitro group meta to the azide. This substitution maintains absorption maximum at wavelengths longer than 280 nm (sparing proteins), and also permits radioiodination of the phenyl ring as opposed to incorporation of a radionuclide into the sugar portion of the molecule. This not only increases the ability to sense the label in low concentrations, but also allows proximity of the radiotracer to the reactive radical. Radiotracer position is important when considering the possibility of hydrolysis of the probe post protein labeling; the amide linkage presents itself as a likely target for hydrolysis, in which case a labeled protein would remain radiolabeled due to the molecular position of the iodine. Derivatives at C-1 and C-6 positions were created because we were also interested in producing probes that would have a high affinity for the transporter from the outside or inside of the membrane, respectively [4].

Recently, several laboratories have attempted to label the glucose transport protein(s) using photolabile transport inhibitors. In studies of cytochalasin B photoinduced binding to red cell membranes, the substance appears to label the 4.5 region on SDS gels [5], a region consisting of at least six bands. Neither reconstitution studies nor cytochalasin B binding data are able to identify which of these bands is related to transport activity. While the highest activity labels the 4.5 region, other neighboring bands also show associated radioactivity. In the investigation of sodium-coupled transport systems, phlorizin photoaffinity probes have been used. These probes exhibit a high affinity in transport experiments, but unfortunately are not very specific and label many bands on SDS gels [6,7].

Our reagent appears to be much more specific than either of these probes; photoactivation studies using erythrocyte membranes as a model system reveal the labeling of two discrete SDS bands (see Results). The synthesis of this probe and others has been described elsewhere [2,3]. We report here characterization studies of the aryl azide C-6-substituted glucose, a high-affinity photoaffinity label for the hexose transporter. Parts of this work have been presented in abstract form [31].

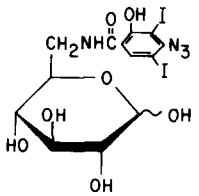

Fig 1 Structure of the C-6-substituted D-glucose aryl azide, 6-*N*-(4-azido-2-hydroxy-3,5-diiodobenzoyl)-D-glucosamine For labeling studies, the ^{125}I probe was prepared

Methods

The synthesis and iodination procedures of the glucose photoaffinity probe (AHDB-GlcN) were carried out as described previously [3]. For incorporation into transport or labeling buffer systems, stock solutions of high concentration were prepared by solubilizing a small quantity of the photoaffinity probe (less than 1 mg) in 15 μl dimethylformamide, to which was added 500 μl 300 mosM 0.5% boric acid, NaCl buffer (pH 7.2) (borate buffer), and 485 μl 5 mM sodium phosphate (pH 8.0) (buffer 1, 5P8). The stock solution was then diluted to the appropriate concentration.

Preparation of resealed ghosts. Resealed ghosts for labeling studies were made in buffer 1 plus 1 mM Mg^{2+} (buffer 2) as described by Steck [8]. Freshly drawn human red blood cells were washed in phosphate-buffered saline (pH 8.0) and subjected to hypotonic lysis in buffer 2. After three washes, the ghosts were stored at 4°C until use.

Ghosts used in transport studies were sealed in borate buffer as follows. Phosphate-buffered saline washed red cells were lysed in buffer 1, and resuspended in the borate buffer, and incubated 2 h at 37°C to promote resealing. The ghosts were subsequently washed three times and stored at 4°C until use.

Transport studies. Initial rates of D-glucose uptake at room temperature in the dark were measured using a rapid uptake centrifugation technique. A 2-s time-point was chosen to be indicative of initial rates since the uptake of D-glucose was found to be linear for up to 5 s at all concentrations of glucose tested. 20 μl (18 μg protein) aliquots of borate-sealed ghosts as prepared above were incubated for 2 s with an equal volume of borate buffer containing a known quantity of D-[U-^{3}H]glucose (31 1 Ci/mmol, New England Nuclear, Boston, MA), with or without the nonradioactive iodinated photoaffinity reagent. Short time-points and precise timing were possible because the radioactive solution could be placed in the bottom of the tube, while the suspension of ghosts would remain beaded on the side of the polycarbonate tube until transport was initiated by vortexing. A metronome emitted audible signals at second intervals, and transport was terminated by repipette injection of 1.25 ml ice-cold phloretin stopping solution (1 mM $HgCl_2$/0.1 mM phloretin/150 mM NaCl). The samples were centrifuged in an Eppendorf tabletop centrifuge for 3 min, the supernatant aspirated and pellets washed in an additional 1.25 ml stopping solution. Pellets were dissolved in Aquasol II (New England Nuclear, Boston, MA) and counted in a Beckman liquid scintillation counter (LS-100, Beckman Instruments, Piscataway, NJ) The average of four determinations was taken as one observation, a zero value was subtracted and velocities were calculated per second. The zero time-point was derived by adding ghosts directly to the unlabeled stopping solution and labeled glucose. At each concentration of glucose studied, an equilibrium uptake value was determined to insure that initial rates were being measured.

Photoactivation studies. Samples for photoactivation were loaded into 300-μl wells of a 96-well tissue culture plate, cut down to 12–20 well sizes compatible with our experimental needs. The culture plate on ice was placed 10 cm away from a high-intensity Hanovia 200 W quartz mercury-vapor lamp having a pyrex 280 nm cutoff filter and a boroscilicate water-jacket (Ace Glass, Vineland, NJ) through which tap water was circulated. The temperature surrounding the lamp was never greater than 2°C above the room temperature.

For the spectrophotometric studies, 150 μM aliquots of the photoaffinity reagent dissolved in 0.04% dimethylformamide and 0.5% borate buffer were exposed to the light for 30, 60, 120 and 240 s time periods. The sample was then scanned in a Cary 118C dual-beam scanning spectrophotometer (Varian Associates, Palo-Alto, CA) between 200 and 400 nm.

Membrane samples for labeling studies followed by electrophoresis (usually 150 μg protein) were combined in titer plates with the appropriate concentration of AHDB-GlcN in dimethylformamide/borate as above, as well as any other protective agent. These samples were exposed to light for 1 min, diluted with 500 μl borate buffer, and centrifuged in an Eppendorf centrifuge for 3 min. The pelleted material was washed with an additional 1.0 ml buffer, centrifuged again and prepared for gel electrophoresis.

For the multiple exposure labeling experiment, membrane samples were labeled as above, using 25 or 2.5 μM concentration of the probe, washed, and then reexposed one to eleven times more with fresh reagent. Extensive washing was performed before each reexposure. After the last photolysis, final protein concentration was determined, the samples were solubilized in Gammascint (National Diagnostics, Somerville, NJ) and counted in a liquid scintillation counter to measure incorporation of iodine.

SDS-polyacrylamide gel electrophoresis and autoradiography. Gels were prepared according to the method of Laemmli [9]. To each sample was added an equal volume of SDS sample buffer, the samples were boiled for 2 min and loaded on 10% slab gels. Gels were stacked at 70 V and usually run at 125 V through the separating gel until completion. The Coomassie blue-stained gels were dried and placed against Kodak X-AR film and DuPont Quanta III intensifying screens (National X-Ray Products, Clinton, NJ) in a −70°C freezer. After the appropriate exposure, films were developed with a Kodak HRP developer for 4 min.

Results

A two-pronged approach was used to characterize the ability of the photoaffinity reagent to probe glucose transport: (1) by testing its ability to

inhibit glucose transport function in red cell ghosts, and (2) by performing labeling experiments designed to identify a specifically labeling species that could be saturated and protected by glucose and other known transport competitive inhibitors. In order to begin our studies, special consideration needed to be given to the problem of solubilizing a substance designed to be hydrophobic.

Borate has been described as useful in the solubilization of highly insoluble substances having available hydroxyl groups [10]. In particular, the borate-complexing behavior of sugars has long been recognized [11] and used in chromatographic separation of sugars. When used in membrane studies utilizing highly hydrophobic probes, the boronate complex that is formed exists in equilibrium, theoretically dissociating at the cell membrane. For these reasons, 0.5% boric acid was used in our buffers to allow complete solubilization of the reagent. If the complex did not dissociate, atypical D-glucose transport kinetics would be observed. To test this, borate-sealed ghosts were prepared and the initial rate of D-glucose uptake was measured, using a rapid uptake procedure to assay 2-s time-points (described above).

The K_m for D-glucose uptake in our system was calculated to be 1.98 mM, while V_{max} was 28 nmol/s per mg protein (not shown). These values are consistent with those reported by Lacko et al. [12] in influx studies, and others by other techniques [13,14]. Therefore, the boronate complex did not interfere with transport and probably was dissociating at the membrane.

Once this was established, kinetic experiments were performed in the absence of activating ultraviolet light to test the reagent's ability to inhibit the transport process. Fig. 2 is a Dixon plot of experiments carried out at various concentrations of glucose and the mass spectra analyzed iodinated probe. It is apparent from this graph that the photoaffinity reagent did indeed inhibit glucose uptake, and in addition: (1) inhibition was dose-dependent and could be classified as of the competitive type, (2) the inhibitor had a high affinity for the transport process, revealing a K_i value in the range of 0.07–0.2 μM, and (3) glucose in the medium was protective, decreasing the degree of inhibition at greater concentrations (where AHDB-GlcN concentration was constant).

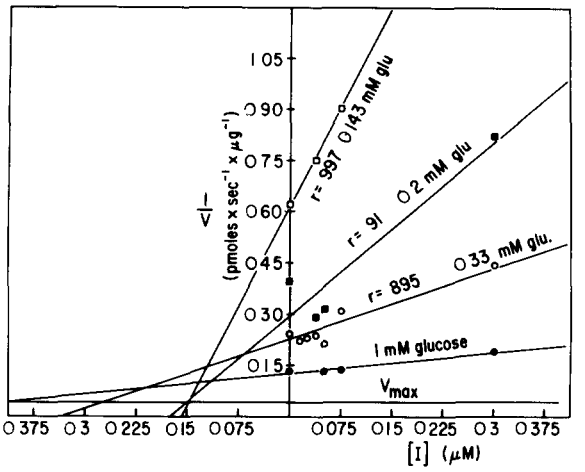

Fig 2 Dixon plot of initial rates of D-glucose uptake experiments performed in the dark in the presence of differing concentrations of the AHDB-GlcN Each point represents the average of four observations V_{max} represents a value derived from control kinetic experiments. The convergence and intersection of lines at each glucose concentration with V_{max} gives the K_i of a competitive inhibitor (between 0 07 and 0 2 μM)

Having shown an affinity of the photoaffinity reagent for the glucose transporter, labeling studies were performed with the light-activated probe. To establish conditions for optimum labeling, the amount of light exposure necessary for photoactivation was determined as well as the concentrations and conditions which would reduce nonspecific binding while enhancing specific binding.

Fig. 3 summarizes the results of a spectral study of the shift in absorbance maxima in the photoaffinity reagent when exposed to high-intensity ultraviolet light for different lengths of time. A shift from 332 nm for the unreacted product to 310 nm for the light-exposed product is apparent, the conversion essentially complete after 1 min of exposure at this concentration (108 μM). In addition, the appearance of an isosbestic point indicates that a single reactive intermediate is produced by light activation. When compared to autoradiograms of labeled samples exposed for different lengths of time, 1 min of exposure seemed to effect more prominent labeling than shorter time-points, but was not significantly different from samples exposed for 4 min. In addition, samples exposed to light for 1 min did not differ

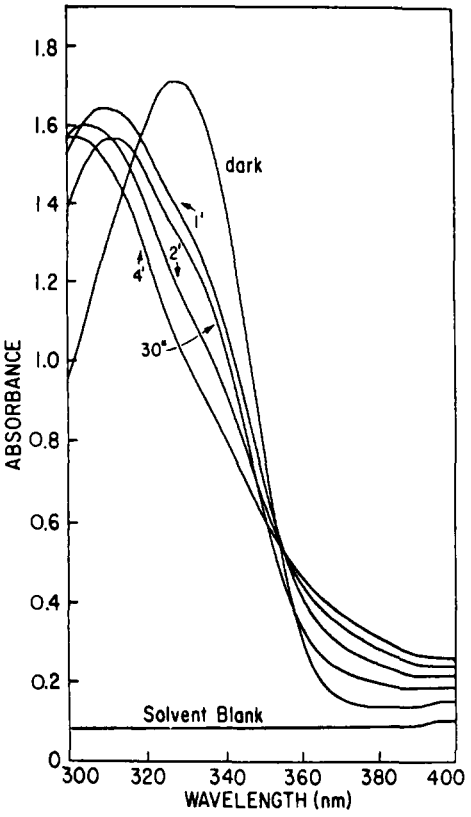

Fig 3 Scanning spectrophotometry of 108 μM AHDB-GlcN exposed to a high-intensity mercury-vapor lamp for different time periods

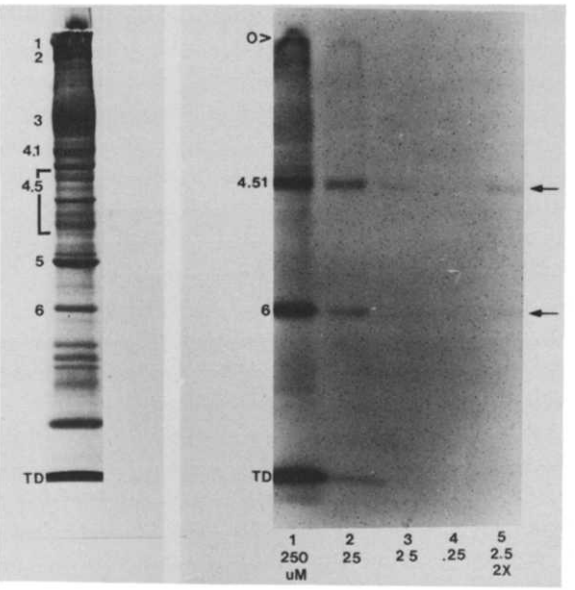

Fig. 4 Labeling of erythrocyte ghosts by light-activated AHDB-GlcN. (A) Coomassie blue-stained proteins run on 10% SDS-polyacrylamide gels with Steck numeric band identifications (B) Autoradiograms of samples exposed to different concentrations of the AHDB-GlcN· lane 1, 250 μM; lane 2, 25 μM; lane 3, 2 5 μM; lane 4, 0.25 μM; lane 5, exposed to 2.5 μM twice Arrows indicate the position of Coomassie staining bands 4.51 and 6.

in transport activity from unexposed samples (not shown). 1 min of light exposure was used for all further labeling studies.

Once the time of exposure was established, concentrations of the AHDB-GlcN that would label transport proteins without promoting nonspecific labeling were determined. Fig. 4 shows the results of a labeling experiment performed at four different concentrations of the ^{125}I-labeled photoaffinity reagent. When compared to the Coomassie blue-stained lanes on 10% gels, labeling could be seen of bands 4.51 and 6, as designated by the nomenclature of Steck [15]. This labeling was not visible at 0.25 μM, but could be seen at concentrations of 2.5 μM and above. At high concentrations (250 μM), other proteins appear to be nonspecifically labeled and the intensity of bands 4.51 and 6 are the greatest.

It has been suggested that multiple exposures of membranes to a photoaffinity reagent could increase specific yet minimize nonspecific labeling. That is, light activation in the presence of a low concentration of reagent, washing and addition of that same concentration of fresh reagent and subsequent light exposure will enhance specific labeling. Lane 5 of Fig. 4 shows no nonspecific labeling and greater labeling of the bands after two exposures than was achieved in a single exposure. This was the rationale for an experiment in which saturation of specific sites was attempted by multiple exposures of ghosts to two different concentrations of the photoaffinity reagent.

When multiply exposed samples are assayed for their incorporation of iodine, with each successive exposure of ghosts to 2.5 μM AHDB-GlcN, the incorporation per μg protein increased linearly. Multiple exposures of the ghosts to 25 μM concentrations resulted in incorporation which appeared to be asymptotic. The data from these two ranges of concentrations were also seen to converge when a sample exposed 11 times to 2.5 μM

AHDB-GlcN was compared to a sample exposed once to 25 μM reagent. From the apparent saturation, the number of glucose carriers could be determined by taking into account the specific activity of the probe assuming that: (1) only a single protein site was labeled and (2) that free probe was not solubilized in the lipid moiety of the membrane. Both of these factors would tend to increase the apparent number of binding sites seen. The measured number of sites is about $2 \cdot 10^6$ molecules/cell. This corresponds very closely to the number of carriers calculated from our transport kinetic data ($3.4 \cdot 10^6$ sites/cell) using a turnover number of 0.366 cm/s and other assumptions as described by Jones and Nickson [16]. While this is consistant with other transport-derived values sited in this same review, data from cytochalasin B binding studies indicate approx. a 10-fold lower number of binding sites. Since our probe, for as yet unexplained reasons, labels glyceraldehyde-3-phosphate dehydrogenase and a substantial amount of labels is seen at the front of SDS gels, it is likely that the amount of 4.51 labelling represents less than one-third of the total label.

While we were able to show transport inhibition with AHDB-GlcN and the labeling of two proteins – one of which is in the region that has been implicated by others as a protein involved in the transport process – a more conclusive identification would be possible if one or both of the labeling bands could be protected by the inclusion of the substrate or a transport inhibitor in the medium. To perform this experiment, concentrations of the protection agents approx 20-times their K_m (or K_i) values were used to compete with a concentration of AHDB-GlcN greater than 25-times its K_i (Fig. 5). By the use of the following equation [17], we were able to calculate the expected percent inhibition:

$$\%I = \frac{[I]}{[I] + K_i(1 + ([S]/[K_m]))}$$

Substituting the values used in our experimental system, under strictly competitive conditions we would expect 1% inhibition, or 99% protection. By light activation, the nature of the competition of the photoaffinity probe is changed from a competitive reversible association to an irreversible one, which would be expected to decrease the observed degree of protection. In addition to a control performed in the absence of protection agents, a

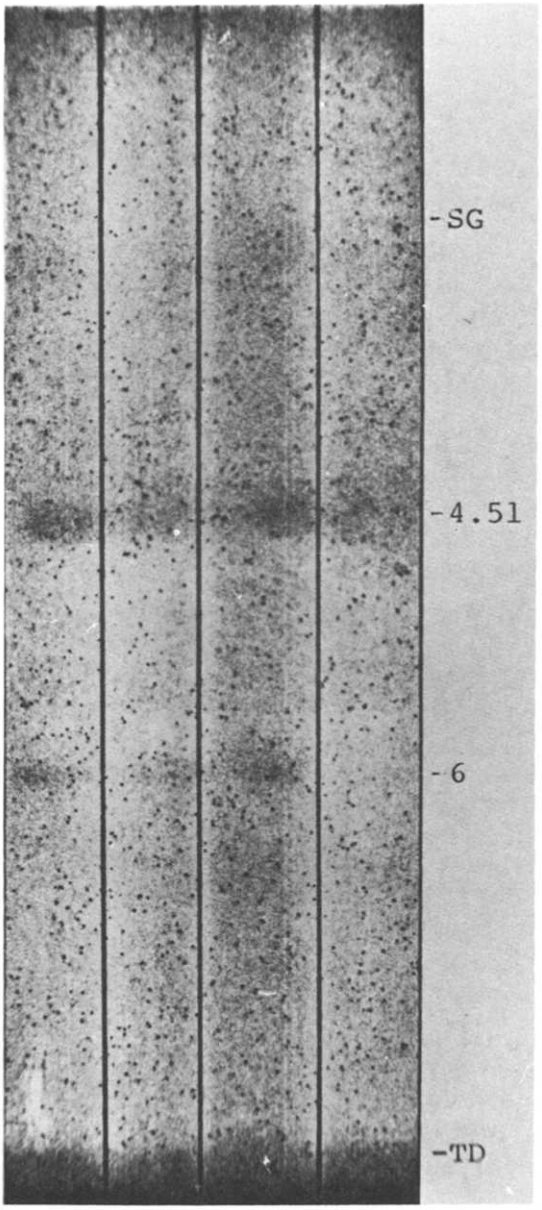

Fig 5 Autoradiography of ghosts labeled in the presence of protection agents Membranes were exposed to 25 μM AHDB-GlcN (from left to right) in the absence (control) or in the presence of D-glucose, L-glucose, or phloretin Gel positions are noted by SG (stacking gel), TD (tracking dye), and 4 51 and 6 (Coomassie staining bands 4.51 and 6)

control sample was included which was exposed to AHDB-GlcN plus 40 mM L-glucose, an isomer of the substrate which is not transported via the hexose carrier. A comparison of the control in the absence of protecting agents (lane 1, Fig. 5) with the sample having an equal concentration of AHDB-GlcN plus 40 mM D-glucose (lane 2, Fig. 5) reveals a noticeable decrease in the density of binding to band 4.51, showing a protection by D-glucose. L-glucose, however, did not protect this site (lane 3, Fig. 5), revealing no difference from lane 1. When this experiment was performed in the presence of 0.1 mM phloretin (lane 4, Fig. 5), it was surprising to note that in addition to protection of the 4.51 site, binding was completely blocked at band 6. Although absolute quantities of protein loaded on the gel for each sample were not determined, it was possible to compare relative densities as a percentage of total radioactivity (data not shown). Examination of densitometry traces revealed a ratio of 1.2 for band 4.51/6 labeling in lanes 1 and 3, while lane 2 ratio was 0.62. Band 6 labeling in lane 4 could not be determined by the densitometer.

Discussion

In order to characterize a potential photoaffinity probe, an assessment of its ability to compete for the transport process as well as the study of its affinity and specificity for the transporter is necessary. Since the transport system of the red cell is probably the best understood experimental system, it was chosen as a model.

Initial transport experiments performed in subdued light were designed to assess the reagent's native ability to compete for the glucose transporter. When initial rates were examined it could be seen (Fig. 2) not only that the reagent was inhibitory and had a high affinity for the transporter, but in addition, transport could be protected by excess glucose in the medium. A K_i value in the range of 0.07–0.2 μM was obtained for the probe. While a precise value is difficult to measure, since the experiment is performed under minimal light conditions, an affinity of AHDB-GlcN greater than 2000-times the affinity of glucose for the carrier was observed. This indicated that the synthesized reagent was a powerful inhibitor, and had great potential of acting as a successful labeling agent. In addition, the nature of inhibition could be identified as competitive, verified by Cornish-Bowden kinetic analysis (not shown). The strictly competitive nature of the probe, however, does not preclude the reaction of the label with other nonspecific sites upon light activation. The probe can be used as a radiolabel of transporters only if specificity of the light-activated process is demonstrated.

It appears that with this reagent, high affinity was matched by a high specificity. While nonspecific labeling was apparent in high concentrations, lower concentrations of 25 μM and less labeled only two SDS-polyacrylamide gel electrophoresis bands (Fig. 4). The labeling at high concentrations of the probe may either be due to nonspecific associations which may be reduced by the addition of scavengers, or to photolytic loss of iodine. An additional source of nonspecific labeling may be long lifetime of the intermediate relative to the time it spends in the active site. Nonspecific problems, however, are not encountered when low concentrations are used in labeling studies. In addition, it is likely that the probe penetrates the membrane in high concentrations since nonspecific labeling of intrinsic membrane proteins can be observed (250 μM, Fig. 4). The intact nature of these ghosts is demonstrated by the fact that [^{14}C]glucose accumulation can still be measured (not shown). It remains to be established whether penetration of the reagent is due to its transport via the carrier, or a penetration due to lipid solubility.

Specific labeling of proteins by low concentrations of AHDB-GlcN resulted in the labeling of two proteins designated 4.51 and 6. The identity of band 6 is known as the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); enzymatic assay of labeled membranes indicated a dose-dependent loss of activity (not shown). Labeling of this band, however, was noted to vary in relative intensity from preparation to preparation.

To our knowledge, glyceraldehyde-3-phosphate dehydrogenase has never functionally or physically been linked to the red cell glucose transporter. However, association of this and other glycolytic enzymes with band 3 has been suggested by kinetic

experiments performed by Kliman and Steck [18]. In addition, Solti and co-workers [19] have visualized this enzyme's localization at the inner surface of the red cell plasma membrane. More recently, a specific peptide of band 3 has been identified as the glyceraldehyde-3-phosphate dehydrogenase binding site [20]. It is possible that band 6 has some physical relationship to the glucose transporter and in this way is seen to label.

Subsequent study in our laboratory of the interaction of AHDB-GlcN with purified rabbit muscle glyceraldehyde-3-phosphate dehydrogenase revealed a high affinity of the chemically blocked AHDB-GlcN precursor, not the final product, for this enzyme (Hertford, S., unpublished data). Isopropylidene protective groups on the adjacent hydroxyls of the precursor renders this compound structurally similar to glyceraldehyde. HPLC analysis of the final labeling product, however, verifies that even in the absence of this precursor, band 6 labels. Further study is necessary to determine the nature of band 6 labeling and to examine the mechanism of phloretin protection.

The labeling of a band in the 4.5 region, however, was expected, as numerous investigators have implicated the 4.5 region as being responsible for glucose transport [21–23]. While the transporter has been isolated and reconstituted, specific identification of a protein in the 4.5 region from these studies is difficult. In studies of photoinduced cytochalasin B covalent labeling, this is also true. It appears that the labeling region is quite broad, despite the existence of discrete Coomassie blue staining bands. Although cytochalasin B has been used successfully in quantitation studies of the glucose transporter, it may be ill-suited as a photolabel due to its lack of specificity, and its apparent inefficient light absorption.

The mechanism by which cytochalasin B is capable of covalent modification is unknown; however, the compound seems to be relatively light-insensitive, requiring high-intensity light activation times of up to 9 min [24]. This is in contrast to short exposure times necessary to activate highly reactive azides often used as labeling agents, and 1 min of exposure required in other systems.

A lack of specificity is evidenced by curvilinear Scatchard plots of cytochalasin B binding in red cells, interpreted to be the result of three high-affinity binding sites, two of which may be unrelated to the glucose-inhibitable site [25]. In order to linearize and calculate binding data, quantification experiments are performed by the addition of other cytochalasins to saturate sites unrelated to transport, as well as protection of the glucose site by 500 mM glucose [26]. The existence of these multiple high-affinity sites is another probable cause of the diffuse binding observed in photolabeling studies, in spite of the use of protective agents.

The labeling observed in our studies, however, is quite distinct and discrete. Competitive transport inhibition observed in the presence of AHDB-GlcN in the dark and significant protection of labeled 4.51 by D- (not L-) glucose and phloretin in the medium is highly supportive of this protein's identity as a transport protein.

While AHDB-GlcN has been prepared in an ^{125}I-labeled form, the specific activity obtained (mCi/mmol) has been much less than is theoretically possible (Ci/mmol). Unfortunately, this low specific activity has made our detection difficult, and efforts are underway to increase the specific activity of the reagent in order to probe systems which have far fewer carriers available on the membrane. In addition, we are exploring further the possibility of deriving accurate quantitative data on the numbers of glucose carriers as this seems to be a central issue in systems where glucose transport activity is modulated In response to insulin [27], starvation [28], exercise [29] and viral transformation [30], a significant increase in the rate of transport occurs. Under some of these conditions, an increase in V_{max} has been observed when comparing the basal and modulated states. This reagent may enable the visualization of these differences and offer a means of probing critical factors in the molecular mechanism of transport and transport regulation. The reagent is also likely to serve as an excellent tool to probe the active site of a disrupted isolated transporter molecule.

Acknowledgements

The authors wish to thank Steven Hertford for glyceraldehyde-3-phosphate dehydrogenase experiments performed, and HPLC analysis of photoaffinity reagents. In addition, the support of NIH

grant AM 17874 for a portion of this work is graciously acknowledged.

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