Photoaffinity Labeling of the Human Erythrocyte Monosaccharide Transporter with an Aryl Azide Derivative of \( \text{d-Glucose} \)*

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A photoreactive, radiiodinated derivative of glucose, \( N-(4\text{-iodoazidosalicyl})-6\text{-amido-6-deoxyglucopyranose} \)(IASA-glc), has been synthesized and used as a photoaffinity label for the human erythrocyte monosaccharide transporter. Photoactivation and photoinactivation are both light-dependent and result in a marked decrease in the absorption spectra of the compound. When \( [^{125}\text{I}]\text{IASA-glc} \) was photolyzed with erythrocyte ghost membranes, photoinertion of radioactivity was observed in three major regions, spectrin, band 3, and a protein of 58,000 daltons located in the zone 4.5 region. Of the three regions which were photolabeled, only labeling of polypeptides in the zone 4.5 region was partially blocked by \( \text{d-glucose} \). In the non-iodinated form, \( [^1^2^6^1^]\text{IASA-glc} \) was photolyzed with \( \text{d-glucose} \) transporter in conjunction with enzymatic probes (11, 12). While all of the above methods have provided useful information concerning the transporter, they also possess a number of limitations. These limitations, which vary with the method employed, include such things as low specificity, high nonspecific background labeling, low efficiency of incorporation, and low specific activity of radiotracer in the compounds being used.

Many of the problems encountered with photolabeling can often be overcome by the use of an orthiodophenylazide functional group on the photoaffinity probe (13). In the present study we describe a simple method for the synthesis of a phenyl azide derivative of \( \text{d-glucose} \), \( N-(4\text{-azidosalicyl})-6\text{-amido-6-deoxyglucopyranose} \)(ASA-glc) and its carrier-free radiiodinated derivative \( [^{125}\text{I}]\text{ASA-glc} \). The results presented in this report show that \( [^{125}\text{I}]\text{ASA-glc} \) can be used for specific covalent labeling of the human erythrocyte monosaccharide transporter.

EXPERIMENTAL PROCEDURES

Materials—\(^{125}\text{I} \)was from New England Nuclear. Electrophoresis reagents and molecular weight markers were purchased from Bio-Rad, except for Sepharose grade grade sodium lauryl sulfate which was from Pierce Chemical Co. 6-Amino-6-deoxy-\( \beta \)-glucose hydrochloride was purchased from United States Biochemical Co. \( N\)-Hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA) was obtained from Pierce Chemical Co. All other reagents were purchased from Sigma. Thin layer chromatography was performed using Whatman type K5F silica gel plates for both analytical (type LK3DF) and preparative (type PLK5F) procedures. Outdated blood was provided by the American Red Cross Regional Blood Center, Madison, WI.

Preparation of Plasma Membranes—Washed human erythrocytes and membrane ghosts were prepared by the method of Steck and Kanta (14). Ghost membranes in 5 mm sodium phosphate buffer (5P8 buffer) were used in the same day for photolabeling experiments. The pellets were isolated by centrifugation at 35,000 \( \times \) \( g \) for 40 min and washed twice with 5P8 buffer. All procedures were carried out at 4 °C unless noted otherwise.

Synthesis of ASA-glc—6-Amino-6-deoxy-\( \beta \)-glucose HCl (85.6 mg, 0.4 mmol) was dissolved in 2.5 ml of dimethyl formamide followed by the addition of 55.3 pl (0.4 mmol) of triethylamine to neutralize the solution. NHS-ASA (120 mg, 0.44 mmol) was added to the mixture and stirred for 48 h at room temperature with constant stirring. The reaction mixture was allowed to proceed for 48 h at room temperature with constant stirring. At the end of this time the dimethyl formamide was removed by vacuum drying. The oily residue was dissolved in 1.5 ml of absolute ethanol and further purified by preparative thick layer (1-mm) chromatography on silica gel plates with butanol:acetic acid:water (5:1:1). The major UV-positive band (\( R_f \approx 0.75 \)) was removed and extracted 4 times with 2 ml of ethanol using a scinttered-glass funnel. The solvent was removed from the combined extracts on a rotary evaporator under reduced pressure. The residue was washed in 2 ml of methanol/benzene (1:3, \( v/v \)) and centrifuged. The supernatant was removed, its volume was reduced to 1 ml by drying, and it was centrifuged again. The white solids collected were combined and dried under \( N_2 \); yield, 90%; m.p. 183-185 °C; IR (KBr): 2143 cm\(^{-1}\) (azide) and 3400 cm\(^{-1}\) (broad —OH); NMR (deuteromethyl sulfoxide): \( \delta \) 6.05 (t, 1H, aromatic H para to hydroxyl), \( \delta \) 6.25 (2H, aromatic H ortho to hydroxyl), \( \delta \) 7.05 (d, 1H, aromatic H meta to hydroxyl).

Synthesis of \( [^{125}\text{I}]\text{ASA-glc} \) —Iodination of ASA-glc was performed using the method of Hunter and Greenwood (15). 10 \( \mu \)l of 0.1 N HCl

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was added to a vial containing 10 μl of Na<sup>125I</sup> (1 mCi) in 10 μl of 0.1 M NaOH, followed by the addition of 20 μl (100 nmol) of ASA-glucose solution (1.7 mg/ml in 0.5 M sodium phosphate buffer, pH 7.4). To this solution 20 μl of chloramine-T (1.14 mg/ml in phosphate buffer) was added and the reaction was allowed to proceed for 1 min, followed by quenching with 50 μl of 5% sodium metahisulfite. Initial purification of [125I]ASA-glucose was performed by thin layer chromatography using ethyl acetate/isopropyl alcohol/water (65:22:11, v/v). The radioactive product was identified on the plate by autoradiography and extracted from the silica gel 4 times with 1 ml of 95% ethanol. The volume of the extract was reduced to 100 μl by drying under N<sub>2</sub>. ASA-glucose (125 μg) was added and the mixture was rechromatographed in butanol/acetic acid/water (5:1:1, v/v). A subsequent autoradiogram indicated that the radiiodinated product migrated slightly ahead of the UV-positive ASA-glucose. This established the separation of [125I]ASA-glucose from ASA-glucose. The radiiodinated product was removed from the plate, extracted 4 times with 1 ml of ethanol and the volume reduced to a final concentration of 0.05 μCi/μl by drying under N<sub>2</sub>. The product was obtained carrier-free at a theoretical specific activity of 2200 Ci/mmol.

**Results**

**Photodecomposition of ASA-glucose.** An 80 μM aqueous solution of ASA-glucose was exposed to various times of irradiation: a, control; b, 3 s; c, 5 s; d, 10 s; and e, 30 s. Samples were then placed in 3-ml quartz cuvettes (1-cm) and the scanning absorbance spectra was determined.

pellets were then solubilized in gel electrophoresis sample buffer (5) and samples (80–100 μg of protein) were layered onto gels for electrophoresis. Under our present photolysis conditions, no detectable photocriss-cross-linking of membrane proteins to each other is evident for up to 30 s.

**Electrophoresis—** Electrophoresis was performed on 12% gels using the modified Laemmli buffer system of Giulian et al. (16). Following electrophoresis, gels were fixed, stained, sliced, and assayed by scintillation counting as previously described (4). For analysis of [125I]ASA-glucose photolabeling experiments, stained gels were first dried on a slab-gel dryer, then exposed to Kodak X-Omat film with a Cronex Hi-Plus intensifier screen (DuPont) at −100 °C for the time indicated. Autoradiographs were scanned using a Bio-Med (Fullerton, CA) 504XL Soft Laser Scanning densitometer (633 nm) with Apple 2e computer integration. Peak heights for gel lanes from the same experiment were normalized using spectrin band heights to account for slight differences in load and nonspecific labeling. The Coomassie Blue protein staining patterns for these red cell polypeptides were typical for this gel system. These staining patterns were essentially unchanged for both control and treated membranes following photolysis.

**Other Procedures—** Protein determinations were performed according to the modified Lowry procedure reported by Peterson (17).

**RESULTS**

**Photodecomposition of ASA-glucose.** In order to produce a photactive analog of D-glucose suitable for photoaffinity labeling of the erythrocyte glucose transporter, we used a heterobifunctional photoactivatable compound, NHS-ASA, originally reported by Ji and Ji (18), coupled to 6-amino-6-deoxyglucosamine to form ASA-glucose. This compound was then radiiodinated using the Hunter-Greenwood method (15). The synthesis scheme and structures are presented in Fig. 1. The optical absorption spectra following light-sensitive activation of the aryl azide is shown in Fig. 2. A substantial photosensitivity is
exhibited by this compound as illustrated by the decrease in the absorption spectra with increasing time of photolysis. The 270 nm band rapidly disappears during the first 5 s of irradiation.

Labeling of the Transporter with \( [^{125}\text{I}]\text{ASA-glc} \)—Fig. 3 shows the autoradiograph (and its densitometric scans) of a NaDodSO₄-PAGE gel of human erythrocyte plasma membranes following incubation with \( [^{125}\text{I}]\text{ASA-glc} \) in the presence or absence of D-glucose. Some label is visible migrating in the region of the tracking dye at the bottom of the gel in both lanes of the autoradiograph (Fig. 3A). In addition, in the absence of glucose two dark bands and one light band are visible following irradiation of the membranes with the photolabel (Fig. 3A, upper lane). The band near the top of the gel corresponds to the region of spectrin. A second faint band is visible around 90 kDa in the band 3 region. The third band which is labeled is in the region of zone 4.5 and corresponds to a \( M_r \) of around 58,000. When D-glucose was included prior to photolysis, labeling of the 58-kDa band was significantly decreased, with only a small decrease in labeling of the high molecular mass region (Fig. 3A, lower lane). A densitometric scan of these two lanes is presented in Fig. 3B. Integration of the peaks after normalization to the spectrin peak height (see "Experimental Procedures") indicates that D-glucose (480 mM) inhibits photoincorporation of IASA by approximately 49% in the 58-kDa region.

Experiments were also performed to determine if ASA-gluc would inhibit photoincorporation of \( [^{125}\text{I}]\text{ASA-glc} \) into the transporter region. The results are shown in Fig. 4. The upper lane of Fig. 4A represents photolabeling of the membranes by \( [^{125}\text{I}]\text{ASA-glc} \) in the absence of ASA-glc, while the lower gel represents membranes labeled under the same conditions but in the presence of ASA-glc (10⁻⁵ M). The densitometric scans of both gels are shown in Fig. 4B. Integration of the areas under the 58-kDa peaks (normalized to spectrin as described...
under "Experimental Procedures") indicates that ASA-glc inhibits incorporation of label by 51% in the transporter region with little or no effect on incorporation into the other regions of labeling.

**DISCUSSION**

The results of experiments presented in this report suggest that \(^{125}\text{I}\text{ASA-glc}\) is a useful photoaffinity probe of the human erythrocyte monosaccharide transporter. Photoactivation of this compound resulted in its covalent incorporation into erythrocyte membrane polypeptides, as evidenced by the fact that the compound is not displaced under the harsh denaturing conditions of NaDodSO\(_4\)-gel electrophoresis. Furthermore, at low concentrations, IASA-glc specifically labels regions of labeling. This photolabel should prove to be a useful tool for further probing both structural and functional aspects of the monosaccharide transport system in the human erythrocyte and possibly other cell systems.

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**Note Added in Proof**—Since the submission of the present report for publication, a study using a similar approach has been published (Weber, T. M., and Eichholz, A. (1985) *Biochim. Biophys. Acta* **812**, 503–511). These authors report that a diiodinated aryl azide derivative of D-glucose (19) competitively inhibits glucose transport in intact erythrocytes. In addition, following photolysis this compound specifically labels bands 4.51 and 6 in concentration dependent, D-glucose protectable manner.

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