PHOTOAFFINITY LABELING AND IDENTIFICATION OF (A COMPONENT OF) THE SMALL-INTESTINAL Na^+,D-GLUCOSE TRANSPORTER USING 4-AZIDOPHLORIZIN

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Received 23 June 1981

1. Introduction

We report here the identification of (a component of) the rabbit small-intestinal Na^+,D-glucose membrane transporter. It is a polypeptide of apparent M_r 72 000. The reagent used is 4-azidophlorizin (fig.1, II), which in the dark has proved to be a fully competitive inhibitor of both Na^+-dependent D-glucose transport and Na^+-dependent phlorizin binding in this membrane [1].

2. Materials and methods

Unlabeled phlorizin was purchased from Carl Roth, Karlsruhe (FRG). Low levels of phloretin in the commercial product were removed by silica gel column chromatography, using methylene chloride–methanol, 4:1 (v/v) as the eluent, and the glucoside was recrystallized from warm water. The synthesis of *para*-phlorizin (phloretin 4′-glucoside; fig.1, III) has been described before [2].

The photoreactive 4-azidophlorizin and its ^3H-labelled analogue were synthesized and chemically characterized as described elsewhere [1]. 4-[^3H]-Azidophlorizin had a specific activity of 0.405 Ci $mmol^{-1}$ and according to the tritiation procedure used (TR-7, Amersham Corporation, Arlington Heights, IL) tritium was introduced into the aglycone moiety by catalytic exchange of benzylic hydrogens.

Reagents for polyacrylamide gel electrophoresis and Coomassie Brilliant Blue R-250 were purchased from Serva, Heidelberg (FRG). Sephadex G-10 was supplied by Pharmacia Fine Chemicals. All other reagents were of highest quality available.

Protein was determined according to Lowry et al. [3], using bovine serum albumin as the standard.

Brush border membrane vesicles from fresh scrapings of rabbit small intestine were prepared by the Ca^{2+}-precipitation method [4], as modified [5]. The vesicles were resuspended in 300 mM mannitol, 10 mM Tris–HCl (pH 7.0) and 0.02% KN_3 (Buffer A) (2–4 mg protein ml^{-1}) and extracted with potassium deoxycholate (DOC), as described in [6], except that a slightly higher concentration of DOC was used (0.65 mg DOC mg^{-1} protein instead of 0.5). After extraction the membranes were centrifuged at 60 000 × *g* for 30 min, and the pellets were washed once with buffer A. Treatment of DOC extracted membranes with 0.6 M KI was according to [7].

Photolysis of membranes in the presence of 4-[^3H]-azide was performed as follows: membranes (1.5 mg protein ml^{-1}) previously deoxygenated with argon for 30 min according to [8] were incubated with ^3H-label and appropriate additions in a medium containing 300 mM mannitol, 100 mM NaSCN (out, zero in), 10 mM *p*-aminobenzoate (pABA)/Tris (pH 7.0), 1% ethanol and 0.02% KN_3 for 10 s in the dark. Subsequently the reaction mixture was irradiated in a water-jacketed quartz cuvette (at a distance of 30 cm) for 1 min at 20°C with a 350 W mercury arc lamp (Illumination Industries Inc., Sunnyvale, CA) equipped with a 3 cm liquid filter of saturated Cu(II)SO_4 to cut

Abbreviations used: DOC, deoxycholate; SDS, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, tris-(hydroxymethyl)-aminomethane; PAGE, polyacrylamide gel electrophoresis; K_i', inhibition constant of analogues as determined from D-glucose uptake, and referred to the total (ionized + unionized) concentration of the analogue [14]

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off light below 315 nm. Following irradiation, the samples were diluted three fold with ice cold buffer A and immediately applied to a Sephadex G-10 column (0.8 × 4 cm) equilibrated with 300 mM mannitol, 100 mM KCl, 10 mM Hepes/Tris (pH 7.5) and 0.02% KN_3 to remove free ^{3}H-label and photoproducts [9]. After washing by centrifugation the membranes were solubilized for 60 s at 95°C in sodium dodecyl sulfate (SDS) sample buffer (4% SDS, 12% glycerol, 12% β-mercaptoethanol, 0.002% bromphenol blue, 10 mM Tris–HCl, pH 7.0; final concentrations).

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Lämmli [10], as modified [11]: 50-μg samples of protein were applied onto a 2-mm thick discontinuous slab gel with an 8.4% (w/w) acrylamide running gel. The electrode buffer was 0.025 M Tris, 0.3 M glycine and 0.1% SDS. The gel was run for 12 h at room temperature, applying a constant current of 4 mA cm^{-2}. Gels were stained in 0.1% Coomassie Brilliant Blue R-250, destained, and photographed with a Polaroid Land camera.

Fluorography was performed as in [12], using PPO and dimethylsulfoxide. A mixture of ^{14}C-methylated proteins of selected relative molecular masses was used to calibrate the gel. Gels were dried and exposed to a Kodak X-Omat R film for 50 days at −70°C.

In order to determine the radioactivity associated with band 'T', the band was cut from a PPO-impregnated, dried gel, treated with NCS tissue solubilizer (Amersham Corporation, Arlington Heights, IL) according to [13] and counted.

3. Results and discussion

The identification of the Na^+, D-glucose transporter in the small-intestinal or renal brush border membranes has turned out to be a formidable and complex task. The reasons for this are probably many fold, but at least two are clear: carrier density is very low in the natural membrane (approximately 10–100 pmol mg^{-1} protein, [14–16]) and the transporter has a poor stability when solubilized in detergents (S. and Y. Takesue, unpublished data, 1979). Yet, some progress has been achieved by negative purification [6], by semi-selective labeling with $HgCl_2$ [17], and by solubilization and reconstitution [18–22]. The limitations inherent in each of these approaches are either self-evident or have been pointed out by the authors.

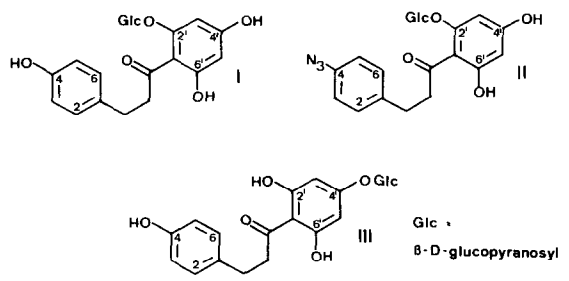

Fig.1. The structures of phlorizin (I), 4-azidophlorizin (II) and *para*-phlorizin (III).

Additional strategies include affinity and photoaffinity labeling. Neither, to the best of our knowledge, has ever been applied successfully to this system, although the latter approach is a powerful method and offers some promise. Potential labels of the Na^+, D-glucose transporter should clearly be designed with phlorizin (fig.1, I) as a model, since this ligand has the highest or second highest affinity known for this transporter [23,24]. Elsewhere we have reported the synthesis and use of phlorizin derivatives substituted at position 6 of the D-glucopyranosyl moiety [24]. In the present paper we report the successful labeling of the small-intestinal Na^+, D-glucose transporter using 4-azidophlorizin (fig.1, II).

One of us has proposed that hydrogen bonding between the hydroxyl group at position 4 of ring B (acting as the acceptor) and the corresponding site on the Na^+, D-glucose transporter is an important contribution to the overall interaction energy [23]. Nevertheless, the photoreactive azido group was introduced into position 4, since the specificity requirements of the interaction with the binding site does allow some modification in this position. Furthermore, we surmized that substitution of the hydroxyl group with an azido group would still permit some hydrogen bonding at this position during the dark reaction, as azido groups have been reported to accept hydrogen bonds [25]. However, we did find a limited loss in binding energy (in the dark, approximately 1.7 kcal mol^{-1}, [1]).

4-Azidophlorizin was indeed found to be an effective, fully competitive inhibitor of Na^+-dependent D-glucose transport and of phlorizin binding to the carrier in subdued light [1]. It was thus to be expected that the reactive intermediate(s) generated by photolysis under appropriate conditions may bind covalently to the Na^+, D-glucose transporter.

Clearly, non-specific labeling, i.e., labeling of other components of the brush border membrane must also be expected. Some important requirements must be met, therefore, before a band in a SDS–PAGE pattern can be identified as the Na^+, D-glucose transporter or a part thereof. Accordingly, in planning the representative experiment shown in fig.2, the following independent criteria were applied for differentiating:

(i) The band must be labeled at relatively low concentrations (e.g., 100 μM for a K_i' of approximately 110 μM at pH 7.0 (K_i' at pH 6.5, 92 μM; K_i' at pH 7.5, 140 μM [26].)) of 4-azidophlorizin, under experimental conditions known to be conducive to optimal binding of the ligand and, of course, to activation of the azido group (see section 2).

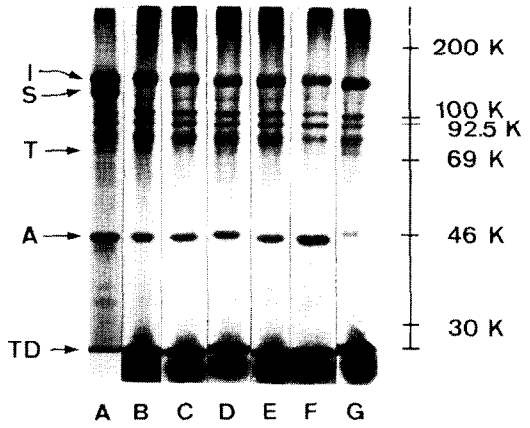

Fig.2. Fluorograms showing the covalent labelling of small intestinal brush border membranes by 4-[^{3}H]azidophlorizin. The membranes were incubated, irradiated, washed, solubilized in SDS under reducing conditions, and 50-μg samples of protein were analyzed by SDS-PAGE as described under section 2. Illustrated are a Coomassie blue stain of DOC-extracted membranes (A) and the fluorograms (B to G) of DOC-extracted membranes (B,C,D,E and G) or of native brush border vesicles (F), which have been photolabeled in the presence of 100 μM 4-[^{3}H]azidophlorizin: B: azidophlorizin alone; C: azidophlorizin plus 200 μM phlorizin; D: azidophlorizin plus 200 μM *para*-phlorizin; E: as in B, but in the presence of choline SCN (100 mM), instead of NaSCN; F: native brush border membrane vesicles, azidophlorizin alone; G: DOC-extracted, KI-extracted membranes, azidophlorizin alone. Some of the bands are designated according to [6], as indicated at the left margin, i.e.: I, isomaltase-rich band; S, sucrase-rich band; A, actin-like protein. The scale of the apparent molecular weights is given at the right margin. The following ^{14}C-methylated proteins were used as standards: myosin (M_r = 200 000), phosphorylase *b* (M_r = 100 000 and 92 500), bovine serum albumin (M_r = 69 000), ovalbumin (M_r = 46 000), carbonic anhydrase (M_r = 30 000).

(ii) The labeling of band 'T' (see fig.2, lane B) should be prevented or reduced by other ligands known to compete with 4-azidophlorizin (e.g., by phlorizin, see fig.2, lane C), but not by other compound(s) which, although chemically related, have little or no affinity for the transporter (e.g., para-phlorizin, fig.1. III, see fig.2, lane D). This control is particularly important, because prevention or reduction of labeling by phlorizin may have been due, also, to optical shielding (absorption) and/or to a scavenging effect of phlorizin, rather than to competition for the same binding site on the transporter. *para*-Phlorizin has the same absorption spectrum (at the pH used) and nearly identical properties as phlorizin, but, unlike phlorizin, has about 100 times lower affinity for the Na^+, D-glucose transporter (K_i' approximately 420 μM [24], see also [27,28], in which somewhat different conditions were used.). Thus, the reduction in the labeling of band 'T' (approx. M_r 72 000) by phlorizin (fig.2, lane C), but not by *para*-phlorizin (fig.2, lane D), strongly suggests that this band is identical with (a part of) the transporter. Note that this differential effect of phlorizin and *para*-phlorizin on labeling by 4-azido-phlorizin is not apparent in any band other than band 'T'.

(iii) The presence of Na^+ is necessary for the interaction of phlorizin with the carrier, as measured via inhibition of D-glucose transport [29] or via [^{3}H]-phlorizin binding [14,16]. Likewise, Na^+ is needed for the interaction of 4-azido-phlorizin with the carrier in the dark [1]. Accordingly, in the absence of Na^+, no photolabeling of band 'T' by 4-azidophlorizin is observed (fig.2, lane E).

(iv) As pointed out above, the Na^+, D-glucose transporter is a minor component of this brush border membrane (approx. 20 pmol mg^{-1} total protein, [6]). Under the conditions used, 2–5 pmols of label mg^{-1} total protein were incorporated into band 'T'. A figure in this range would indeed be expected, considering that the concentration of 4-azidophlorizin was not saturating, that the labeling yields with nitrenes are generally low [8], etc.

(v) Procedure(s) known to lead to a negative purification of the Na^+, D-glucose transporter should also lead to a similar purification of band 'T'. This is indeed the case: (a) DOC extraction: The labeling of the band 'T' is considerably stronger with DOC extracted membranes (fig.2, lane B) than with intact

membrane vesicles (fig.2, lane F). In the latter the labeling of band 'T' is indeed barely detectable. (b) KI extraction: DOC extracted membranes can be further treated with KI, which removes the majority of the cytoskeleton proteins [7], without inactivating or releasing the Na^+, D-glucose transporter [6]. Accordingly, the labeling of band 'T' remains constant or even increases in KI-treated, DOC-extracted membranes (fig.2, lane G, as compared to lane B). (It must be mentioned that the treatment of intact membrane vesicles with 0.6 M KI did not result in a detectable release (and thus decrease in the labeling) of cytoskeletal elements, such as actin (see fig.2, band A). This suggests that the actin labeled with intact membrane vesicles (fig.2, lane F) is located inside sealed membrane vesicles.)

Thus, band 'T' is in all likelihood the Na^+, D-glucose transporter or a component thereof. Its apparent M_r (approx. 72 000) agrees with previous indirect conclusions of the laboratory in Zurich from partial negative purification [6] or semi-selective labeling with $HgCl_2$ [17]. The figure obtained is compatible also with those reported by other laboratories from solubilization-reconstitution of kidney cortex membranes (60–70 000 Da, under denaturing conditions, [22], or approximately 160 000 Da, under non-denaturing conditions, [20]).

It is possible from the identification of band 'T' with (a part of) the Na^+, D-glucose transporter to draw some conclusions on its nature and its mode of functioning. A first one is neither novel nor surprizing: as band 'T' has an apparent M_r of approx. 72 000 and stains with Coomassie blue (fig.2, lane A) it is in all likelihood proteinaceous. Secondly, a polypeptide chain of 72 000 Da can (although admittedly need not) span the membrane, a feature for which some evidence has been presented [30,31]; it is large enough to build (a part of) a gated pore.

The mode of functioning of this membrane transporter, indeed, that of all 'mobile carriers', is still a matter of speculation. Since it shows a stable structural [17,30,31) and functional [32] asymmetry, a 'rotational' or 'diffusive' mechanism can be ruled out. Whether monomeric or oligomeric, the most likely mode of operation seems to be that of a 'gated channel' [33, see also 34] which is a limiting case of membrane transporters [35].

Finally, the fact that a reactive group at position 4 of ring B does indeed react with the Na^+, D-glucose carrier allows some inference to be made on the mode of phlorizin's (and of its derivatives') interaction with it. The K_i'-value of phlorizin for D-glucose transport and the K_d'-value of phlorizin binding to brush border membrane vesicles is approximately 4–7 μM [14,36]. These values are considerably smaller than the K_m values of monosaccharides or most β-glucopyranosides for transport which lie in the mM range (the smallest value being perhaps that of D-glucose 'initial' uptake into brush border vesicles, 0.1 mM [37]. Also, the K_i value of the only monosaccharide known to us to act as a fully competitive inhibitor (L-fucose, in intestinal rings, 20 mM [38]), is much larger than that of phlorizin. Therefore, in order to explain the higher binding energy of phlorizin one of us suggested [23] that this glucoside binds to the carrier both at its sugar-binding site (via the glucopyranosyl moiety) and in addition at a (presumably more hydrophobic) site (via the aglycone moiety). The subsites interacting with the aglycone moiety of phlorizin could in part be lipids. The observations of fig.2 do not rule out the occurrence of lipids at the aglycone binding site(s), but they clearly demonstrate that a reactive group in position 4 of ring B can react covalently with a polypeptide.

It is possible that the aglycone binding site may interact with free phloretin [23,39], for which there is also some indirect kinetic evidence [39,40].

Acknowledgements

The present work was partially supported by the SNSF, Berne, and by the National Institute of Health, Bethesda, Maryland, (Grant AM 06878).

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