# Identification of the D-glucose binding polypeptide of the renal $Na^+$-D-glucose cotransporter with a covalently binding D-glucose analog

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The covalently binding D-glucose analog 10-*N*-(bromoacetyl)amino-1-decyl-β-D-glucopyranoside (BADG) was synthesised and shown to be a high-affinity inhibitor of the renal $Na^+$-D-glucose contransporter. From renal brush-border membranes a protein fraction was isolated, in which the concentration of $Na^+$-dependent phlorizin binding sites per mg protein was enriched 7-fold. In labeling experiments with this protein fraction a polypeptide of $M_r \sim 79000$ was identified as containing the D-glucose binding site of the renal $Na^+$-D-glucose cotransporter.

*Affinity labeling* *D-Glucose analog* *D-Glucose binding site* *$Na^+$-D-glucose cotransporter* *Brush-border membrane* *Kidney*

## 1. INTRODUCTION

Identification and purification of the $Na^+$-D-glucose cotransport proteins from kidney or small intestine is a difficult task. Whereas purification attempts did not yet lead to conclusive information concerning the identity of the transporter proteins [1–5], experiments with side group specific protein reagents and monoclonal antibodies suggest that a polypeptide with a molecular weight of about 72000 may be a component of the $Na^+$-D-glucose cotransporter from small intestine [6,7]. Data obtained with the photoaffinity reagent 4-azidophlorizin [8] suggest that the 72-kDa polypeptide from intestine possibly contains the D-glucose binding site. Here, the synthesis of the covalently binding D-glucose analog BADG is reported. With this reagent the D-glucose binding site of the renal $Na^+$-D-glucose cotransporter, which is supposed to be structurally different from the D-glucose transporter from intestine [9], could be identified on a polypeptide of $M_r$ 79000 ± 4000.

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*Abbreviations:* BADG, 10-*N*-(bromoacetyl)amino-1-decyl-β-D-glucopyranoside; TRA buffer, 10 mM triethanolamine-HCl (pH 7.4), 150 mM EDTA disodium salt, 10% (v/v) glycerol; KC buffer, 20 mM imidazole cyclamate (pH 7.4), 0.1 mM magnesium cyclamate, 100 mM potassium cyclamate; NaC buffer, 20 mM imidazole cyclamate (pH 7.4), 0.1 mM magnesium cyclamate, 100 mM sodium cyclamate; TLC, thin-layer chromatography; DTT, dithiothreitol

## 2. MATERIALS AND METHODS

Bromo[1-$^{14}$C]acetic acid (58 mCi/mmol) was supplied by Amersham Buchler (Braunschweig). Chemicals used for synthesis were of reagent grade and were purchased from Merck (Darmstadt). All other chemicals were of highest grade and were obtained as described earlier [4,5].

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2.1. *Synthesis of 10-N-(bromo[$^{14}$C]acetyl)amino-1-decyl-β-D-glucopyranoside (BADG)*

The synthesis of 10-amino-1-decanol from sebacic acid has been previously described [10–12]. The amino group was protected by a benzyloxycarbonyl group as, e.g., described in [13], and the product was recrystallized from chloroform-light petroleum (40–60°C) and characterized by TLC. To form the glycosidic bond 9.86 g of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide in 30 ml ethanol-free chloroform were added to a mixture of the protected aminodecanol (8.29 g), silver carbonate (5.54 g), iodine (240 mg), molecular sieves 4 Å (30 g) and dry ethanol-free chloroform (120 ml) and stirred for 1 day in the dark [14]. The reaction mixture was filtered through Celite and the solvent was evaporated. The residue was dissolved in chloroform/methanol (99/1, v/v) and purified on a silic acid column which was run with the same solvent. The compound was deacetylated in dry methanol containing 0.03 N sodium methoxide. 10-Benzyloxycarbonylamino-1-decyl-β-D-glucopyranoside was purified on a silic acid column which was performed with methanol/chloroform (85/15, v/v). To remove the benzyloxycarbonyl group 1 g of the compound was hydrogenolyzed in 20 ml of 0.14 N HCl in 60% (v/v) methanol containing 120 mg of palladium on carbon. The resulting aminoglycoside was yielded chromatographically pure by lyophilization of the filtered reaction mixture and was characterized by TLC and paper electrophoresis. To form the bromoacetyl derivative 2.5 mg of bromo[$^{14}$C]acetic acid were converted to the acid chloride by 12 h incubation in 1 ml thionyl chloride at room temperature and the solution was evaporated to dryness (below 40°C). Then a solution of 50 μl water containing 10 mg of the aminoglycoside and 16 mg $KHCO_3$ was added to the residual syrup and the mixture was incubated for 1 h at 0°C. The final product BADG was purified by preparative paper electrophoresis, which was performed on Whatman 3MM paper in 0.1 M acetic acid-KOH (pH 4), and was squeezed out from the paper.

2.2. *Partial purification of the $Na^+$-D-glucose cotransporter*

The labeling experiments were performed with a membrane protein fraction, in which the number of $Na^+$-dependent high affinity phlorizin binding sites per mg protein was 7-fold enriched compared with intact brush-border membranes. Brush-border membranes were isolated from proximal tubules of the outer cortex of pig kidneys [4] and were solubilized with deoxycholate in the presence of 1 M D-glucose and thereafter centrifuged as described in solubilization procedure c in [5]. When in the supernatant the protein and deoxycholate concentration was reduced to 1.5 mg/ml and 0.1%, respectively, and D-glucose was partially removed by 3 h dialysis against glucose-free buffer some of the solubilized proteins precipitated. The aggregated mixed micelles were separated by centrifugation and washed with 20 mM imidazole-HCl (pH 7.4), 0.1 mM magnesium cyclamate, 100 mM potassium cyclamate (KC buffer). To measure the number of phlorizin binding sites the sample was incubated for 5 min at 37°C with different concentrations of radioactively labeled phlorizin in the presence of 20 mM imidazole cyclamate (pH 7.4), 0.1 mM magnesium cyclamate plus 100 mM potassium cyclamate (KC buffer) or 100 mM sodium cyclamate (NaC buffer) and then washed by Millipore filtration with ice-cold KC or NaC buffer. The number of $Na^+$-dependent phlorizin binding sites in the aggregated mixed micelles was 400–600 pmol per mg protein.

2.3. *Labeling experiments*

Labeling was performed without (i) and with (ii) dithiothreitol (DTT) pretreatment. (i) Aggregated mixed micelles were incubated for 2 h at 37°C in KC or NaC buffer containing 14 μM radioactively labeled BADG. Parallel experiments were performed in the absence of glucose or in the presence of 85 mM D-glucose, 85 mM L-glucose, 85 mM β-methyl glucoside or 85 μM phlorizin. (ii) Aggregated mixed micelles were incubated for 10 min at 37°C in NaC buffer containing 3 mM DTT plus 85 mM L-glucose or plus 85 mM D-glucose. Then the sample was 11 times diluted with ice-cold KC buffer and centrifuged for 30 min at 80000 × *g*. Thereafter the pellets were incubated for 2 h at 37°C in NaC buffer containing 85 mM L-glucose plus 14 μM radioactively labeled BADG.

After incubation with BADG the samples were diluted 10-fold with 100 mM cysteine (0°C) and spun down by centrifugation at 80000 × *g*. For gel electrophoresis the pellets were resuspended in

63 mM Tris-HCl (pH 6.8), 8 M urea, 10% glycerol, 2% SDS, 5% $\beta$-mercaptoethanol and for 10 min incubated at 100°C. 500 $\mu$g portions of protein were applied on top of large diameter 10% polyacrylamide rod gels and electrophoresis was performed according to Laemmi [15]. The gels were fixed with trichloroacetic acid, washed with ethanol/acetic acid/$H_2O$ (25/8/67, v/v), stained with Coomassie brilliant blue R 250, scanned at 600 nm and cut into 2 mm slices. The slices were digested and counted for radioactivity.

## 3. RESULTS

When the reversible interaction of BADG with the $Na^+$-D-glucose cotransporter was analysed by measuring the inhibition of initial $Na^+$ gradient-dependent D-glucose uptake rates in proteoliposomes, which were formed as recently described (see LipIIc in [5]), a $K_i$ of 30 $\mu$M was estimated for competitive inhibition by BADG.

To covalently label the D-glucose binding site of the renal $Na^+$-D-glucose cotransporter two strategies were employed. (i) On the one hand, we tried to protect the D-glucose binding site from covalent BADG labeling by addition of D-glucose or phlorizin. (ii) On the other hand, we tried to cleave or protect cleavage of a disulfide bridge, which has been demonstrated to be essential for substrate binding of the renal $Na^+$-D-glucose cotransporter [16,17], and to covalently label the D-glucose binding site thereafter.

(i) Aggregated mixed micelles, which were prepared as described in section 2, were incubated for 2 h at 37°C with BADG in the presence of $Na^+$ or $K^+$. To demonstrate protection of the D-glucose binding site the incubation was performed in the absence of glucose, in the presence of 85 mM L-glucose, 85 mM D-glucose, 85 mM $\beta$-methyl glucoside or 85 $\mu$M phlorizin. From fig.1 it can be seen that some of the proteins were preferentially labeled. Labeling of proteins was identical if it was performed in the absence of glucose or in the presence of 85 mM L-glucose (not shown). In fig.1b BADG labeling in the presence of 85 mM L-glucose or 85 mM D-glucose is shown. In fig.1c the D-glucose protectable labeling is demonstrated. Although high unspecific labeling of some proteins was observed in the presence of D-glucose, D-glucose protection of a 79 ± 4 kDa polypeptide could be reproducibly demonstrated. Some D-glucose protection was observed in the high molecular mass region and it was found that the

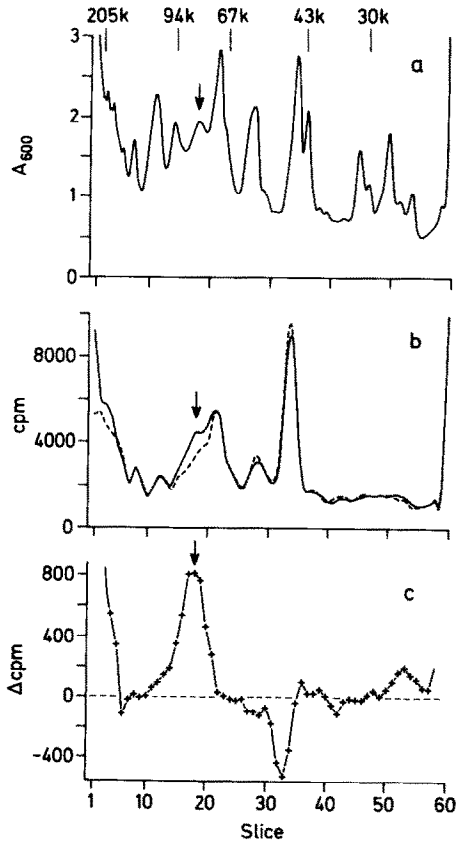


Fig.1. Covalent labeling of renal brush-border membrane proteins with BADG in the presence of L- or D-glucose. Proteinl-lipid aggregates were isolated from renal brush-border membranes and labeled with BADG in the presence of $Na^+$ and either L- or D-glucose. Formation of protein-lipid aggregates, BADG labeling and SDS-polyacrylamide gel electrophoresis are described in section 2. (a) A densitogram of protein-lipid aggregates, which have been separated by SDS-gel electrophoresis, is shown; (b) the radioactivity distribution after BADG labeling in the presence of L-glucose (solid line) or D-glucose (broken line) is demonstrated; (c) the difference between the labeling in the presence of D- and L-glucose is shown. The lines in (b) and (c), which are connecting 54 measuring points, are fitted by computer. In (a) the positions of the marker proteins myosin (205 kDa), phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and carboanhydrase (30 kDa) are indicated.

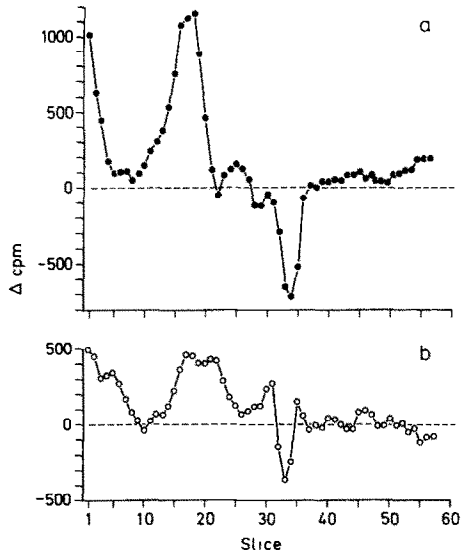


Fig.2. $Na^+$-dependency for phlorizin protection of a 79 kDa polypeptide from BADG labeling. Protein-lipid aggregates, prepared as in fig.1, were incubated with radioactively labeled BADG in the presence of $Na^+$ (a) or $K^+$ (b) as described in section 2. In the graphs the differences between BADG labeling in the presence and absence of 85 $\mu$M phlorizin are shown.

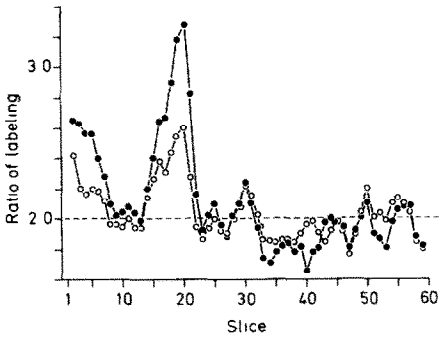


Fig.3. Selective increase of BADG labeling of a 79 kDa polypeptide if disulfides are reduced in the presence of D-glucose. In the presence of D-glucose and $Na^+$ protein-lipid aggregates were incubated with DTT as described in section 2. After washing the samples and in parallel also control samples, which had not been treated with DTT, were incubated with radioactively labeled BADG (in the presence of $Na^+$ and L-glucose). Control labeling was also performed in the presence of D-glucose. In the graph ratios of radioactivity are shown, in which protein labeling after reduction with DTT is related to the protein labeling without DTT pretreatment, performed in the presence of L- (○) or D-glucose (●).

labeling of a 45 kDa polypeptide was slightly increased in the presence of D-glucose (see section 4). In fig.2 the phlorizin protectable BADG labeling measured at pH 7.4 in the presence and absence of $Na^+$ is shown. Since significant protection of the 79 kDa polypeptide was observed only in the presence of $Na^+$ it has been demonstrated that the BADG labeling was reduced if phlorizin bound to the D-glucose binding site. The phlorizin protection of the 79 kDa polypeptide in the presence of $Na^+$ was also observed at pH 6 but was not found at pH 9.5 (not shown).

(ii) When membrane proteins were treated for 10 min at 37°C with 3 mM DTT and then labeled with BADG it was found that labeling of most proteins was increased by a factor of 1.8–2.2 (fig.3). Only in the 79 kDa polypeptide and in the high molecular mass region of the gel was labeling increased to a higher extent. The selective increase in the labeling of the 79 kDa polypeptide by the pretreatment with DTT was more pronounced if control labeling without DTT pretreatment was performed in the presence of 85 mM D-glucose (compare open and closed symbols in fig.3). These data strongly suggest that BADG does not act as an unspecific SH-group modifying reagent but interacts selectively at the D-glucose binding site of the $Na^+$-D-glucose cotransporter.

In fig.4 the difference in BADG labeling of membrane proteins is shown, which had been pretreated with 3 mM DTT in the presence of either L-glucose or D-glucose. It can be seen that due to the presence of D-glucose during DTT pretreatment specific covalently BADG labeling of the 79 kDa polypeptide was observed. Findings of Turner and George [16,17] show that at least two disulfide bridges are cleaved at the D-glucose binding site by DTT and that one of these is essential for substrate binding and can be protected by addition of substrate. Here, the essential disulfide bridge has been cleaved by DTT (presence of L-glucose) or has been partially protected by D-glucose. Selective labeling of the D-glucose binding site by the D-glucose analog BADG was possible in a higher extent if the DTT pretreatment was performed in the presence of D-glucose and more D-glucose binding sites remained intact. The finding that BADG labeling at the 79 kDa polypeptide was

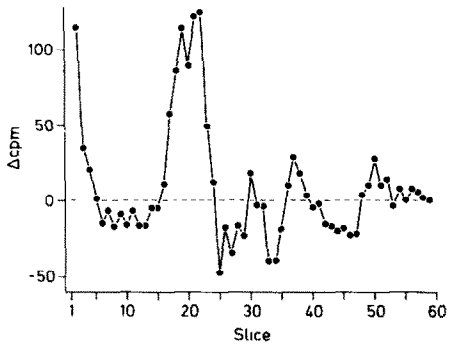


Fig.4. Specific labeling of a 79 kDa polypeptide by BADG if during reduction of disulfide bonds the D-glucose binding site of the $Na^+$-D-glucose cotransporter was protected. Protein-lipid aggregates were in the presence of $Na^+$ plus L- or D-glucose incubated with DTT. After washing the samples were incubated with radioactively labeled BADG (in the presence of $Na^+$ and L-glucose). In the graph the difference of BADG labeling obtained after dithiothreitol pretreatment in the presence of either D- or L-glucose is shown.

increased under a condition in which less SH-groups are generated but more D-glucose binding sites remained intact (presence of D-glucose during dithiothreitol pretreatment) is another indication that BADG labeling is a specific reaction at the D-glucose binding site of the $Na^+$-D-glucose cotransporter.

## 4. DISCUSSION

Similar to other aliphatic glucosides [18] BADG has a higher affinity to the glucose transporter than D-glucose. At variance to photoaffinity labeling reagents [8] BADG reacts with nucleophiles, especially with SH but also with imidazole and amino groups, and is suitable to detect alterations in accessibility or occurrence of these groups. The degree of labeling is supposed to be higher with bromoacetyl derivatives than with photoaffinity reagents. In the case of BADG successful labeling may have been achieved since the bromacetyl group is in a mobile way linked to the reversibly binding D-glucose moiety of the molecule. With BADG a broad 75–83 kDa polypeptide band could be identified to carry the D-glucose binding site of the renal $Na^+$-D-glucose cotransporter. Firstly, labeling with BADG could be protected with D-glucose, $\beta$-methylglucoside or phlorizin. This protection was $Na^+$ and pH dependent. Secondly, this band was shown to react specifically with BADG, if during the reduction of disulfide bonds a disulfide bridge essential for substrate binding was protected by addition of D-glucose and $Na^+$. This appears to be specific for the renal $Na^+$-D-glucose cotransporter [9]. The fact that the identified polypeptide is a rather broad band in the gel suggests that it is a glycoprotein. Since in the high molecular mass region of the highly loaded gels the separation of polypeptides was incomplete (see fig.1a) the D-glucose or phlorizin protectable BADG labeling observed in this region is presumably due to the presence of aggregated 79 kDa polypeptides. In the experiments without DTT pretreatment it was found that in the presence of D-glucose or phlorizin labeling was increased at a 45 kDa polypeptide, which has been shown by immunological studies to be identical with actin (unpublished). This effect could be a reproducible artifact, it may however also be explained by the hypothesis that actin may be closely associated with the transporter, so that D-glucose binding could lead to a concerted structural change which may result in an increase of the unspecific BADG labeling. Target size analysis of $Na^+$-dependent phlorizin binding in renal brush-border membranes revealed two functional units of $M_r$ ~230000 and 110000 [19,20], suggesting that the transporter in the membrane may exist as monomer or dimer. Each monomer, which is able to bind phlorizin, should contain the 79 kDa polypeptide with the D-glucose binding site and possibly another ~30 kDa subunit [21]. Since for the target size of D-glucose uptake in renal brush-border membranes an $M_r$ of ~345000 was estimated [20] it may be speculated that the transport unit may be even more complex. To finally elucidate the molecular structure of the $Na^+$-D-glucose cotransporter the 79 kDa polypeptide and the other hypothetical subunits have to be purified and functionally reconstituted.

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