DEMONSTRATION OF GLUCOSE INHIBITABLE PHLORIZIN BINDING TO RENAL BRUSHBORDER PROTEINS SEPARATED BY GEL ELECTROPHORESIS

Lothar THOMAS
Max-Planck-Institut für Biophysik, Frankfurt/M., Germany

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1. Introduction

The brushborder membrane of the proximal convolution contains a phlorizin binding site, which due to its high affinity for phlorizin and other properties like competitive inhibition by D-glucose and sodium dependence has been suggested to be involved in the transport of D-glucose through the brushborder membrane [1–3]. In order to study the chemical and physical interactions between this receptor and the transported substrate an extraction and purification of the molecule is important. In this paper a procedure is described by which the glucose sensitive phlorizin binding site can be extracted from the membranes in a conformation which still shows the glucose sensitive phlorizin binding. The binding itself is measured by a technique in which after the separation of the proteins by electrophoresis the gel is incubated with [^{3}H]phlorizin and [^{14}C]mannose and after washing the ratio $^3H/^{14}C$ is determined in the gel slices.

2. Methods

The extraction and purification of the glucose sensitive phlorizin binding site from isolated brushborder of rat kidney [4] was achieved as shown in fig. 1.

The gel-electrophoretic separation of the proteins solubilized by papain and deoxycholate treatment was carried out at 2–4° on 11% and 15% acrylamide gels, using the multiphasic borate–sulfate buffer system of Jovin et al. with a running pH of 8.6 [5].

To find out which of the separated proteins in the gel contained the glucose sensitive phlorizin binding site the gels were incubated for 1 hr at 37° with 3×10^{-6} M [^{3}H]phlorizin (1 dpm/10^{-14} moles) in the absence and presence of 0.4 M D-glucose in TRA-HCl–EDTA-buffer (10 mM triethanolamine-HCl, 5 mM EDTA) pH 7.6. The incubation media was further made 3×10^{-6} M with [^{14}C]mannose (1 dpm/10^{-14} moles) and 150 mM with NaCl. [^{3}H]Phlorizin and

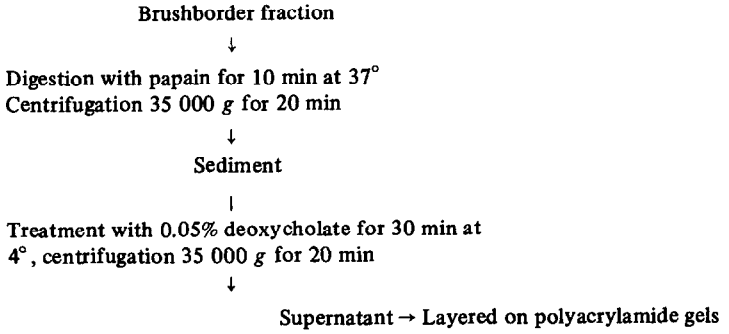

Fig. 1. Extraction and purification of the glucose sensitive phlorizin binding site from isolated brushborder of rat kidney. Digestion with papain (18 EU/27 mg membrane protein) was done in 0.25 M sucrose–0.01 M Tris-HCl 7.6, deoxycholate treatment (0.5 mg/mg membrane protein) in 0.04 M Tris-borate buffer pH 8.6.

[^{14}C]mannose diffuse into the gel and [^{3}H]phlorizin binds to those protein bands containing the phlorizin binding sites. [^{14}C]Mannose in the concentrations used in our experiments did not bind to the gel-electrophoretically separated proteins and was therefore used as correction for the [^{3}H]phlorizin which was solubilized in the gel pores and not bound to protein bands. Therefore an increase of the ratio $^{3}H/^{14}C$ radioactivity in the gel indicated in concordance with the protein stain the proteins which had bound phlorizin.

To determine the radioactivities, the gels with a length of 6 cm and a thickness of 5 mm were inserted in a syringe-like gel fractionator with an internal diameter of 5.5 mm and extruded through a sieve with 600 pores per cm^2 each of 200 μm diameter. By this procedure, the gel was broken into small beads, but extruded to its original form and length. Cylindrical portions of 5 mm length were then put on a nylon net. To reduce the amounts of [^{3}H]phlorizin and [^{14}C]-mannose solubilized in the gel pores, 500 μl unlabeled phlorizin (3×10^{-6} M phlorizin in TRA-HCl–EDTA-buffer) were layered upon the cylindric portion and run through the acrylamide beads by low pressure for 10 sec.

For radioisotope determinations the wet beads of each portion were swollen in 1 ml Soluene® at 50° for 8 hr in a scintillation vial. Then 20 ml of acidified Instagel® were added.

In most of the experiments the wet acrylamide portions were packed in filter paper, dried and combusted in a Tri-Carb Sample Oxidizer, Model 305. Aminopeptidase, alkaline phosphatase and Mg^{2+}–ATPase were determined by incubating the gels with the correspondent substrates [6].

3. Results

After treatment of the brushborder fraction with papain and deoxycholate (fig. 1) the supernatant which was layered on top of the gels contained 8% of the protein, 6% of the alkaline phosphatase and 2% of the aminopeptidase activity of the brushborder fraction. To relate the enzyme activities in the supernatant to those of the untreated brushborder membrane, the effects of the detergents on the enzyme activities were corrected by factors calculated as described [7]. If in 15% acrylamide gel (fig. 2a) the distribution of the

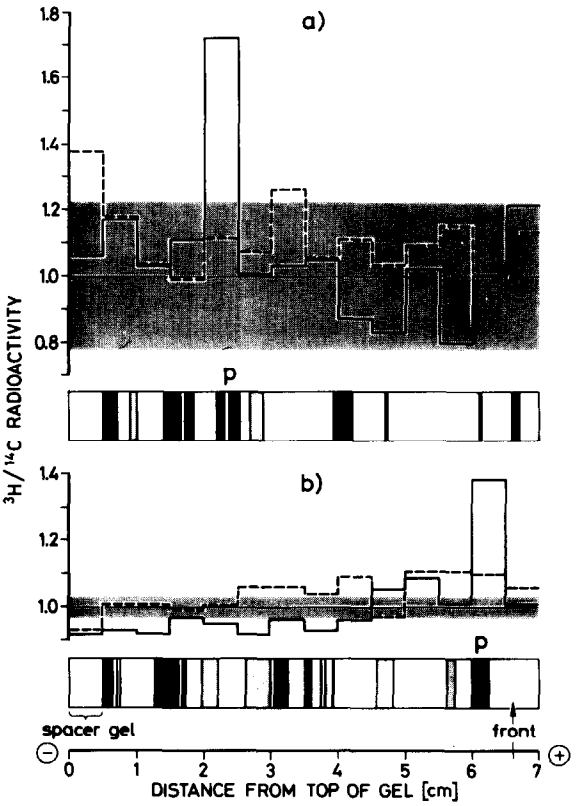

Fig. 2. Disc gel electrophoresis of proteins extracted from brushborder membranes in 0.05% deoxycholate. 50 μg Protein were layered on top of the 15% (a) and 11% (b) acrylamide gels. The protein pattern of the gels stained with Coomassie blue and the pattern of radioactivity of unstained duplicate gels is shown. The gels are incubated with [^{3}H]-phlorizin and [^{14}]mannose in a ratio of 1. Ordinate represents the [^{3}H]phlorizin/[^{14}C]mannose ratio in the gel slices. Abscissa marks the distance from top of the gel. The black bands represent an intensive protein staining, the shadowed ones lower staining. Protein bands containing the phlorizin receptor are marked by P. The standard deviations of $^{3}H/^{14}C$ ratio of slices from gels run without protein are marked by the shadowed areas. The phlorizin binding to the separated protein bands in the absence (solid lines) and in the presence (broken lines) of D-glucose is indicated by an increase in the $^{3}H/^{14}C$ ratio of more than 3× standard deviation. The protein bands which show the strongest phlorizin binding ($^{3}H/^{14}C$ increased) lose their ability to bind phlorizin in the presence of D-glucose ($^{3}H/^{14}C$ decreased).

$^{3}H/^{14}C$ ratio in the phlorizin gel is compared to that gel incubated with phlorizin in the presence of D-glucose, the glucose sensitive phlorizin binding site can be related to a doublet of bands in the protein

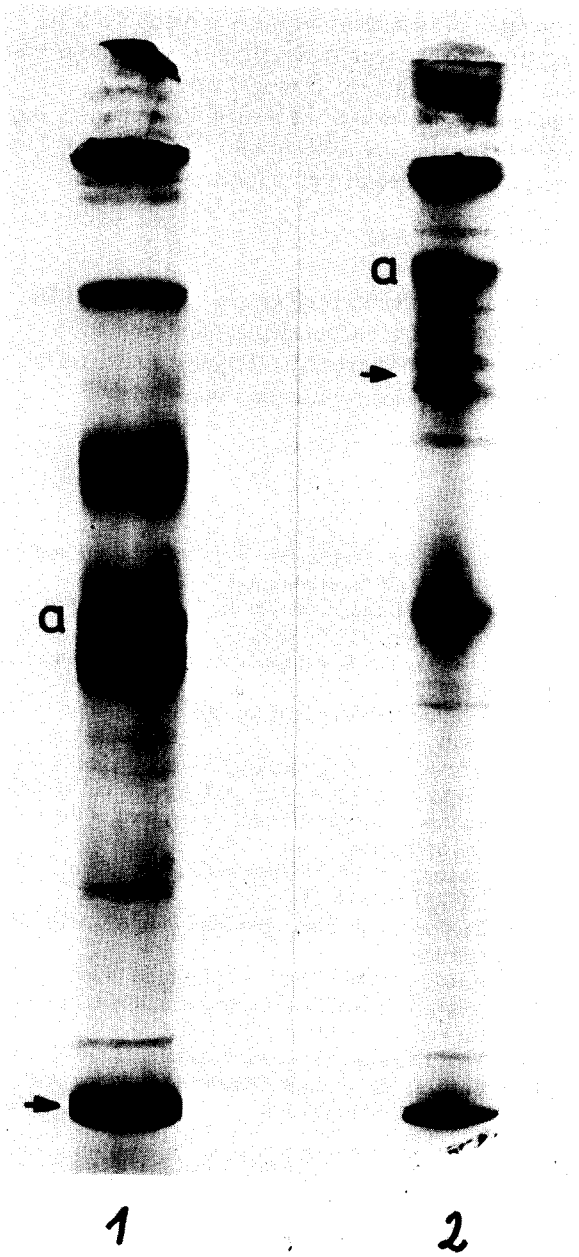

Fig. 3. Polyacrylamide-gel-disc electrophoresis of proteins in 35 000 *g* supernatant after papain and deoxycholate treatment. The protein patterns of 11% gel (1) and 15% gel (2) are shown. The protein bands containing the glucose sensitive phlorizin binding sites are indicated by an arrow. The localization of aminopeptidase activity in the gels is indicated by the letter a. The enzyme activities on top of the gels are not indicated.

pattern. Because the gel is sliced in portions of 5 mm it is not possible to decide whether the phlorizin receptor is localized in both bands of the doublet or only in one.

In 11% acrylamide gels (fig. 2b) the binding site is localized in a protein band just behind the bromthymol-blue front. The shadowed areas, representing the standard deviations of the $^{3}H/^{14}C$ ratios, show that in gels run without proteins or with albumin these ratios vary only slightly from the $^{3}H/^{14}C$ ratio of the incubation medium but is depending on the acrylamide concentration of the gels. The slight variations in the standard deviations show, that there was no preferential uptake of [^{3}H]phlorizin or [^{14}C]mannose into the gel.

The activities of alkaline phosphatase and Mg^{2+}-ATPase were localized on top of the gel. The aminopeptide activity which was found in the gels could not be correlated to the protein bands containing the phlorizin binding sites (fig. 3).

4. Discussion

Essentially two steps were involved in the experiments described in this paper. First the phlorizin receptor had to be extracted in an active form and the phlorizin binding to the purified binding protein had to be demonstrated. Transport proteins, which are localized on the surface of the membranes like the binding proteins isolated from bacterial systems [8] or the sucrase-isomaltase compex from the gut [9] can be extracted easily either by osmotic shock or by treatment of the membranes with papain. The phlorizin receptor however cannot be released by papain because it is localized in the microvillus matrix and not in the glycoprotein coat or in the aminopeptidase containing particles [10]. Therefore detergents have to be used for the desintegration of the complex protein–lipid structure of the membranes. However, the kind of detergent and the incubation procedure is of great importance since by these treatments inactivation of membrane bound receptors can occur. In our hands low concentrations of deoxycholate turned out to be superior to treatment with Triton or dodecylsulfate because binding studies performed in parallel to these experiments showed that the phlorizin receptor is extracted preferentially by deoxycholate

whereby the affinity of the binding sites remaining in the membranes is unchanged [10].

The distribution of the [^{3}H]phlorizin and [^{14}C]-mannose in the acrylamide gels depends on the presence of protein bands. Gel slices containing phlorizin binding proteins show an increase in ^{3}H-count rate but a decrease in the ^{14}C-counts, the ratio ^{3}H/^{14}C increased compared to the ratio in the incubation medium. Gel slices with protein bands that do not bind phlorizin show the same decrease in the ^{3}H- and ^{14}C-count rate and the ^{3}H/^{14}C ratio remains constant.

The amount of phlorizin radioactivity in the protein bands which contain the phlorizin receptor is approximately equal to the phlorizin radioactivity which was bound to the glucose sensitive binding sites of the brushborders before they were treated according to fig. 1. This suggests that by treatment of the membranes with papain and with deoxycholate at pH 8.6 approximately all of the glucose sensitive phlorizin binding sites are extracted.

The separated phlorizin binding site is inhibited by D-glucose, a property which is identical with the behaviour of the untreated membranes [10]. Furthermore, low concentrations (3×10^{-6} M) of phlorizin are used, the receptor purified seems to be identical with the high affinity receptor for phlorizin present in the brushborder membrane and does not represent other low affinity receptors [11, 2].

The glucose sensitive phlorizin binding sites demonstrated in the separated protein bands were extracted with only 8% of the proteins from the brushborders. These proteins can be separated by gel electrophoresis into more than 10 bands. Assuming that the stained doublet in the 15% gels does not contain more protein than the other protein bands the receptor protein represents not more than about 1% of the membrane protein. However exact information can only be achieved if the protein bands containing the binding sites are reelectrophoresed and the receptor is isolated in a pure form.

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