Biochimica et Biophysica Acta 902 (1987) 402–405
Elsevier

BBA 70524

BBA Report

Binding of cytochalasin B to trypsin and thermolysin fragments of the human erythrocyte hexose transporter

A.R. Karim, W.D. Rees and G.D. Holman

Department of Biochemistry, The University of Bath, Claverton Down, Bath BA2 7AY (U K)

(Received 16 March 1987)

Key words Hexose transporter, Cytochalasin B binding, Trypsin, Thermolysin fragment, (Human erythrocyte)

The cleavage of the human erythrocyte hexose transporter by the proteinases trypsin and thermolysin has been studied. When red cell membranes are treated with trypsin, washed and then photolabelled with cytochalasin B, a labelled peak at 18 kDa is obtained. This labelling of the cleaved transporter is D-glucose inhibitable. This probably indicates that the residual 36 kDa portion of the transporter is not required for binding of ligands. Extensive cleavage of the transporter with low concentrations of thermolysin only occurs when transporter is prelabelled with cytochalasin B. This indicates that covalently bound cytochalasin B can cause a conformational change which exposes the thermolysin cleavage site.

It is now clear that erythrocyte band 4 5 is the hexose transporter This protein can be reconstituted into vesicles which show hexose transport [1–3] Band 4 5 binds cytochalasin B [4] and photolabelling studies with cytochalasin B [5–7] and with the azidosalicylate derivative of bis(mannose) ASA-BMPA [8] have identified this protein as the hexose transporter Mueckler et al [9] have obtained the c DNA and corresponding amino-acid sequence for the transporter and the locations of the ligand binding sites at external and internal surfaces of the transporter have been assigned [10] The outside binding site is near tryptophan 363 while the inside binding site is near tryptophan 388 These tryptophan residues are probably responsible for the observed fluorescence quenching induced by the binding of ligands to the transporter [26,27] A hydrogen bonding transport channel has been revealed by proton exchange investigations on purified band 4 5 [23] Minor perturbations of the transporter can occur via interactions in Band 3 [12,13,24]

Cleavage of the transporter by proteinases occurs mainly at the cytosolic surface [14–17] Proteolysis of the transporter has been studied by several groups in order to establish the orientation of the transporter in reconstituted hexose transporter preparations Extensive trypsin digestion is known to abolish transport activity [16,17] and cytochalasin B binding [15] in reconstituted hexose transporter preparations and thus the susceptibility to trypsin action can indicate whether the reconstituted preparation is randomly orientated [17] or mainly right-side out [16]

Deziel and Rothstein [18], Klip et al [25], Shanahan and D'Artel-Ellis [19] and Cairns et al [7] have shown that when the human erythrocyte hexose transporter is covalently labelled with cytochalasin B and subjected to trypsin digestion then a 18 kDa labelled fragment is produced Most of these studies (including the experiments on pro-

Abbreviation ASA-BMPA, 2-*N*-(4-azidosalicoyl)-1,3-bis(D-mannos-4′-yloxy)propylamine

Correspondence G D Holman, Department of Biochemistry, 4-West, The University of Bath, Claverton Down, Bath, BA2 7AY, U K

teinase inhibition of transport function) have used high proteinase concentrations In contrast, however, Cairns et al [7] showed that mild trypsin digestion of over 75% of the purified transporter resulted in only a 50% loss of cytochalasin B binding activity Scatchard analysis showed that the number of binding sites for cytochalasin B was unchanged but that the cytochalasin B equilibrium binding constant increased by about 50%.

We describe in this paper experiments using mild trypsin and thermolysin treatment to cleave the transporter prior to photoactivated labelling with cytochalasin B In the case of trypsin we wished to ascertain whether ligand binding could occur after cleavage We have recently shown [10] that the core of the transporter comprising the outside and inside ligand binding sites plus a possible transmembrane channel are located within the C-terminal half of the protein (hydrophobic spans H7-H10 as defined by Mueckler et al [9]) Since this region is encompassed within the 18 kDa trypsin fragment [11] it seemed possible that this fragment would be fully functional

Erythrocytes from 1–3-week-old transfusion blood were lysed in 5 mM sodium phosphate (pH 7 8) containing 1 mM EDTA and 1 μg/ml PMSF In the thermolysin experiments EDTA was omitted 300 μg of isolated membranes were resuspended in 300 μl of 5 mM sodium phosphate (pH 7 2) in the presence of 10^{-4} M cytochalasin E plus 1 6 μCi of cytochalasin B (15 5 Ci/mmol) Membranes were irradiated for 30 s in a Rayonet Photochemical Reactor (RPR 3000 lamps) in 1-mm pathlength cuvettes The cuvettes were covered with a 10-mm pathlength filter containing 1% cumene in 2,2,4-trimethylpentane Following irradiation the membranes were washed twice in 35 ml of 5 mM sodium phosphate (pH 7 2) and then resuspended at 1 mg/ml for proteinase treatment or solubilised for electrophoresis

Electrophoresis was carried out using a modification of the method of Hashimoto et al [20] using the discontinuous buffer system of Laemmli [21] as previously described [10] The main modification was the use of a gradient of acrylamide (10–15%) and of bisacrylamide (0 5–1 5%) This gave a crosslinking gradient that resolved and retained small peptides but at the same time allowed high-molecular-weight proteins to enter the gel Molecular weight standards were galactosidase (116 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21 kDa), lysozyme (14 kDa), aprotinin (6 6 kDa) and insulin (3 4 kDa)

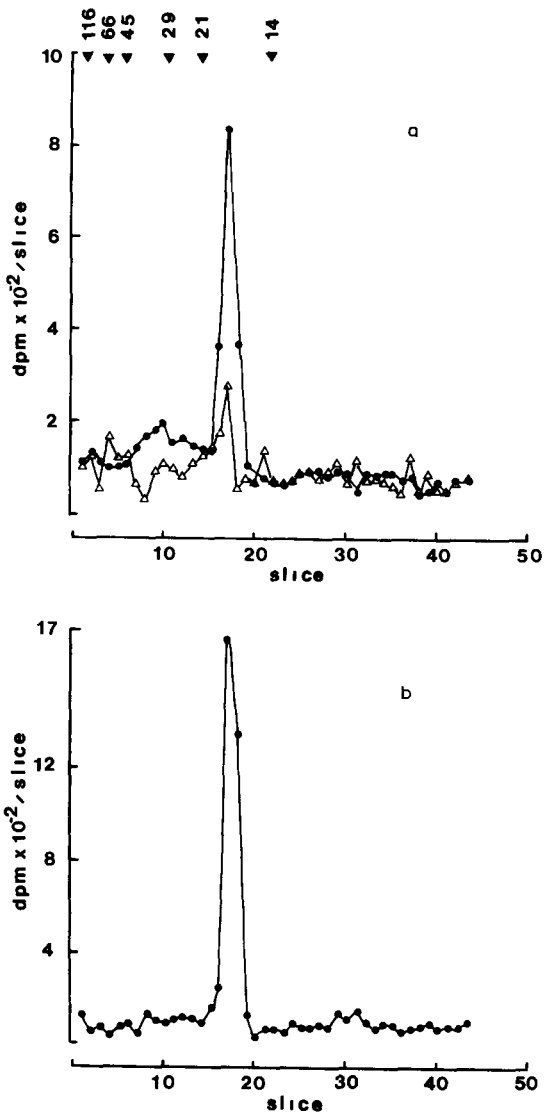

Fig 1 Trypsin cleavage of the hexose transporter (a) before and (b) after labelling with cytochalasin B (a) Membranes were treated with 100 units/ml of trypsin for 30 min at 20 °C After washing, the membranes were labelled with cytochalasin B in the presence (△) or in the absence of 150 mM D-glucose (●) (b) Membranes were labelled with cytochalasin B, washed and then treated with 100 units/ml of trypsin for 30 min at 20 °C

Fig 1b shows a typical pattern of trypsin cleavage of the cytochalasin B labelled transporter A 18 kDa peak is produced In the same experiment membranes were firstly cleaved with trypsin (100 units/ml for 30 min at room temperature) and then labelled with cytochalasin B in the presence or in the absence of 150 mM D-glucose (Fig 1a) The fragment labelled in this way has exactly the same mobility as the fragment that is produced by prelabelling with cytochalasin B and then trypsin digesting As well as being able to bind cytochalasin B the fragment must also be able to bind D-glucose as this displaces the cytochalasin B from the 18 kDa fragment This result suggests that the residual portion of the transporter is not required for binding of ligands

An alternative explanation for our result showing ligand binding after proteolysis could be that proteolysis with trypsin merely 'nicks' the transporter and that the 18 kDa and 36 kDa portions are held together as a functional unit and in their normal conformation during the ligand binding and labelling

Figs 2a, b show a comparison of the thermolysin effect on the cytochalasin B labelled transporter with the photolabelling of thermolysin pretreated membranes The prelabelling with cytochalasin B gives a large acceleration of the thermolysin cleavage and this is consistent with there being a large conformational change when cytochalasin B is covalently attached Kurokawa et al [22] have observed that cytochalasin B photoaffinity labeled hexose transporter elutes from a DEAE column much more slowly than the unliganded transporter They attributed this to an unfolding of an anionic domain upon crosslinking to cytochalasin B It may be that the same conformational change increases the exposure of the thermolysin-sensitive site When the hexose transporter is labelled at its exofacial site with ASA-BMPA there is no significant digestion by thermolysin This is consistent with exofacial ligands causing a conformational change which reduces the exposure of the cytosolic thermolysin cleavage site [10]

In conclusion, the present results support our suggestions for the locations of the external and internal binding sites on the hexose transporter In addition they suggest that the C-terminal half of

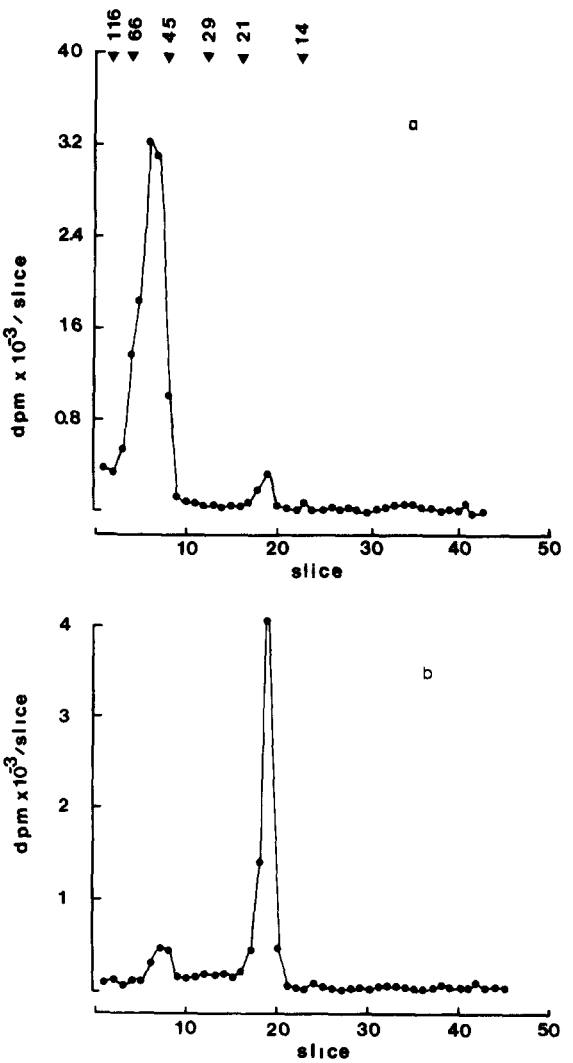

Fig 2 Comparison of thermolysin digestion of the hexose transporter (a) before and (b) after labelling with cytochalasin B (a) Membranes were treated with 0 4 units/ml of thermolysin for 30 min at 20 °C After washing, the membranes were labelled with cytochalasin B Labelling reveals that most of the transporter is not digested with thermolysin (b) Membranes were labelled with cytochalasin B, washed and then treated with 0 4 units/ml of thermolysin for 30 min at 20 °C

the protein which encompasses these sites is fully functional in ligand binding and in an associated conformational change Studies using mild proteinase treatment of the transporter in the presence of noncovalently bound ligands may reveal further details of hexose transport mechanism

We are grateful to the S E R C for financial support

References

1 Baldwin, S A, Baldwin, J A and Lienhard, G E (1982) Biochemistry 21, 3836–3842
2 Kasahara, M and Hinkle, P C (1977) J Biol Chem 252, 7384–7390
3 Carruthers, A and Melchior, D L (1984) Biochemistry 23, 2712–2718
4 Zoccoli, M A, Baldwin, S A and Lienhard, G E (1978) J Biol Chem 253, 6923–6930
5 Carter-Su, C, Pessin, J E, Mora, R, Gitomer, W and Czech, M P (1982) J Biol Chem 257, 5419–5425
6 Shanahan, M F (1982) J Biol Chem 257, 7290–7293
7 Cairns, M T, Elliot, D A, Scudder, P R and Baldwin, S A (1984) Biochem J 221, 179–188
8 Holman, G D, Parkar, B A and Midgley, P J W (1986) Biochim Biophys Acta 855, 115–126
9 Mueckler, M, Caruso, C, Baldwin, S A, Panico, M, Blench, I, Morris, H R, Allard, W F, Lienhard, G E and Lodish, H F (1985) Science 229, 941–945
10 Holman, G D and Rees, W D (1987) Biochim Biophys Acta 897, 395–405
11 Cairns, M T, Panico, M, Morris, H R and Baldwin, S A (1986) Biochem Soc Trans 14, 747–748
12 Rees, W D, Gliemann, J and Holman, G D (1987) Biochem J 241, 857–862
13 Mullins, R E and Langdon, R G (1980) Biochemistry 19, 1199–1205
14 Masiak, S J and LeFevre, P G (1977) Biochim Biophys Acta 465, 371–377
15 Baldwin, J M, Lienhard, G E and Baldwin, S A (1980) Biochim Biophys Acta 599, 699–714
16 Chen, C, Korokawa, T, Shaw, S, Tillotson, L G, Kalled, S and Isselbacher, K J (1986) Proc Natl Acad Sci USA 83, 2652–2656
17 Wheeler, T J and Hinkle, P C (1981) J Biol Chem 256, 8907–8914
18 Deziel, M R and Rothstein, A (1984) Biochim Biophys Acta 776, 1020
19 Shanahan, M F and D'Artel-Ellis, J (1984) J Biol Chem 259, 13878–13884
20 Hashimoto, F, Horigome, T, KanBayashi, M, Yoshida, K and Sugano, H (1983) Anal Biochem 129, 192–199
21 Laemmli, U K (1970) Nature 227, 680–685
22 Kurokawa, T, Tillotson, L G, Chen, C and Isselbacher, K J (1986) Proc Natl Acad Sci USA 83, 479–482
23 Jung, E K J, Chin, J J and Jung, C Y (1986) J Biol Chem 261, 9155–9160
24 May, J M (1987) J Biol Chem 262, 3140–3145
25 Klip, A, Deziel, M and Walker, D (1984) Biochem Biophys Res Commun 122, 218–224
26 Appleman, J R and Lienhard, G E (1985) J Biol Chem 260, 4575–4578
27 Carruthers, A (1986) J Biol Chem 261, 11028–11037