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Photolabelling of the hexose transporter at external and internal sites: fragmentation patterns and evidence for a conformational change

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The human erythrocyte sugar transporter has been labelled at its internal site with cytochalasin B and at its outside site by the azidosalicoyl derivative of bis(D-mannose) (ASA-BMPA). The cleavage of the transporter by various proteinases has been studied. Chymotrypsin, subtilisin and V8 proteinase give parallel fragmentation patterns for the two labels down to fragments as small as 7 kDa. Thus the binding sites for the two labels can only be separated by a small span of protein. 2-Nitro-5-thiocyanobenzoic acid (NTCB) cleaves at cysteines to give a 15 kDa fragment from the two labels. *N*-Bromosuccinimide (a reagent which preferentially cleaves at tryptophan residues) has revealed differences in fragmentation of the transporter labelled with either cytochalasin B or with ASA-BMPA. A major cleavage site is proposed to occur at tryptophan 186 which leaves a C-terminal fragment containing both labels. A tryptophan cleavage at residue 388 divides the cytochalasin B site and the ASA-BMPA site. A further tryptophan cleavage gives a cytochalasin B labelled 3 kDa fragment probably from residues 388–412. This gives an assignment of the cytochasin B site as the inside of the hydrophobic span H 10. Since the ASA-BMPA site is probably only 7 kDa from residue 388 and is on the same 15 kDa NTCB fragment as cytochalasin B we assign this to the outside of hydrophobic span H 9. Thermolysin only cleaves the transporter labelled with cytochalasin B and not with ASA-BMPA. A 18 kDa cytochalasin B labelled fragment is formed. This is indicative of a change in conformation of the transporter when an outside ligand is bound such that the inside of the hydrogen bonding transmembrane segments H 7 and H 8 (and containing the proposed thermolysin cleavage site) are withdrawn from the cytosolic surface. Thus it appears that the core of the transporter (including the external and internal sites plus the transmembrane channel) is located between segments H 7 and H 10.

Introduction

Mueckler et al. [1] have cloned and sequenced a hepatoma cDNA for the sugar transporter. They showed (from chemical studies) that the sequence of the erythrocyte transporter was similar if not identical to that predicted from this nucleotide sequence. This protein (mol. wt. 54117) occurs in the 4.5 band of SDS-polyacrylamide gels of erythrocyte membranes. The availability of this sequence for the transporter is of course a major breakthrough in the investigation of hexose transport and will eventually lead to a full understanding of a molecular mechanism by which the protein catalyses transport. As part of this elaboration of the transport mechanism it is necessary to assign portions of the protein which are likely

Abbreviations: NTCB, 2-nitro-5-thiocyanobenzoic acid; ASA-BMPA, 2-*N*-(4-azidosalicoyl)-1,3-bis(D-mannos-4′-yloxy)propyl-2-amine; PMSF, phenylmethylsulphonyl fluoride.

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candidates for binding substrates and inhibitors at either the external or the internal surface.

Cytochalasin B is thought to bind to an internal site of the transporter [2,3] and can bind covalently in a light-dependent manner [4,5]. When the transporter is covalently labelled with cytochalasin B and then subjected to trypsin digestion a 19 kDa labelled fragment is produced; this fragment is thought to be nonglycosylated [6–8]. Cairns et al. [25] have recently assigned this labelled trypsin fragment to the C-terminus (residues 257–456). This also follows from the observation that the N-terminus contains the carbohydrate attachment site [26]. Cairns et al. [6] also showed that the cytochalasin B labelled protein could be cleaved by the thiol reagent NTCB (2-nitro-5-thiocyanobenzoic acid) into a labelled 15.5 kDa fragment and an unlabelled 38 kDa fragment.

Holman et al. [9] developed the photolabile bis hexose ASA-BMPA (2-*N*-(4-azidosalicoyl)-1,3-bis(D-mannos-4′-yloxy)propyl-2-amine) to specifically label the exofacial surface of the transporter. We have now examined whether the external site labelled by ASA-BMPA is close enough to the cytochalasin B site to give a similar pattern of fragmentation with various reagents and enzymes. Trypsin and chymotrypsin produce a 19 kDa fragment containing the ASA-BMPA label [9]. In addition to a 19 kDa fragment chymotrypsin also produces a number of smaller fragments of around 7 kDa which are common to both the cytochalasin B labelled protein and to the ASA-BMPA labelled protein (Ref. 9 and present authors, unpublished data). We now also show parallel fragmentation patterns for the two labels when the transporter is proteolytically digested with V8 proteinase or with subtilisin or is cleaved at cysteines with NTCB.

Further dissection of the system has, however, revealed differences in fragmentation of internally and externally labelled protein. The use of *N*-bromosuccinimide to cleave at tryptophan residues has allowed us to assign a cleavage point which divides the exofacial site from the internal cytochalasin B binding site. We suggest that the cytochalasin B binding site is in hydrophobic loop 10 while the ASA-BMPA site is on the outside of hydrophobic loop 9. This assignment of the active sites towards the centre of the protein disagrees with the suggestion of Shanahan and D'Artel-Ellis [8] that the cytochalasin B binding site is located at the N-terminus but is in agreement with the recent data of Cairns et al. [25] which indicates that the cytochalasin B labelled trypsin fragment is at the C-terminus.

There is a marked difference in cleavage of cytochalasin B labelled and ASA-BMPA labelled transporter by thermolysin which may indicate that there is a major conformational change in the transporter when ligand is bound at the external rather than the internal site.

Materials and Methods

Materials

2-*N*-(4-Azidosalicoyl)-1,3-bis(D-mannos-4′-yloxy)[2-^{3}H]propyl-2-amine (ASA-BMPA) was prepared as previously described [10,11]. Cytochalasin B and cytochalasin E were from Aldrich. PMSF, subtilisin, thermolysin, 2-nitro-5-thiocyanobenzoic acid (NTCB) and *N*-bromosuccinimide were from Sigma. V8 proteinase was from Miles Laboratories Ltd. [4-^{3}H]Cytochalasin B was from Amersham International.

Cytochalasin B labelling

Fresh erythrocytes or 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. Isolated membranes were resuspended at 300 μg/300 μl in 5 mM sodium phosphate (pH 7.8) 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 demountable cuvettes. The cuvettes were covered with a 1-mm pathlength filter cell 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.

ASA-BMPA labelling

Fresh erythrocytes or erythrocytes from 1–3-weeks-old transfusion blood were labelled with ASA-BMPA as previously described [9]. Follow-

ing irradiation the cells were washed and lysed. Isolated membranes were resuspended at 1 mg/ml in 5 mM sodium phosphate (pH 7.2) and thereafter treated with reagents and enzymes in parallel with cytochalasin B labelled membranes.

Enzyme proteolysis

Membranes at 1 mg/ml were treated with 0.4 units/ml of thermolysin for 30 min at 20°C. The reaction was terminated by washing in 5 mM sodium phosphate (pH 7.2) containing 10 mg/ml EDTA. Treatment with 0.02 units/ml subtilisin was for 30 min at 37°C. Treatment with 86 units/ml V8 proteinase was for 1 h at 37°C at pH 7.8. These reactions were terminated by washing in 5 mM sodium phosphate (pH 7.2).

N-Bromosuccinimide treatment

Labelled membranes were dissolved in a buffer comprising 50 mM sodium citrate (pH 3.0), 1% SDS and 8 M urea to give a protein concentration of 1 mg/ml. *N*-Bromosuccinimide (10 mg/ml) in 10 M urea was then added to a final concentration of 50–500 μg/ml. The reaction was allowed to proceed for 20 min at room temperature and then 2-mercaptoethanol (40 μl/ml) was added. Twice the reaction volume of 0.1 M sodium borate (pH 9.3) was added and the solution was kept at 37°C for 40 min. The samples were desalted prior to electrophoresis by repeatedly filtering through centricon 10 microconcentrators (Amicon corporation) and diluting with a 1 mM Tris-HCl buffer containing 0.1% SDS (pH 6.8). We observed no significant losses of small membrane protein fragments using this procedure.

2-Nitro-5-thiocyanobenzoic acid treatment

Labelled membranes were dissolved in a buffer comprising 42 mM Tris-acetate (pH 8.0), 0.8 mM EDTA, 1.7% SDS and the sample was gassed with nitrogen. NTCB was then added to a final concentration of 10 mM and the reaction was kept at 37°C for 15–30 min. An equal volume of 0.1 M sodium borate (pH 9.3) was added and the pH readjusted to 9.3 by the addition of 1 M NaOH. The mixture was then maintained at 35°C for 24 h. 2-Mercaptoethanol (30 μl/ml) was then added. The samples were desalted as described for the *N*-bromosuccinimide-treated samples.

Electrophoresis

Samples were prepared for electrophoresis by solubilising in a buffer containing 40 mM Tris-HCl (pH 6.8), 8% glycerol, 5% 2-mercaptoethanol and 5% SDS. Molecular weight standards (Sigma) were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine albumin (66 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21 kDa), lysozyme (14.3 kDa), cytochrome *c* (12.5 kDa), aprotinin (6.5 kDa) and insulin (3.4 kDa). Electrophoresis was carried out using a modification of the method of Hashimoto et al. [12] using the discontinuous buffer system of Laemmli [13]. The linear gradient slab gels were 10–15% acrylamide, 0.5–1.5% bisacrylamide in 7 M urea, 0.45 M Tris-HCl (pH 6.8), 0.1% SDS and 0–10% sucrose. The crosslinking gradient was used to improve resolution and retention of small peptides. The stacking gel contained 5% acrylamide, 0.13% bisacrylamide, 0.67 M Tris-HCl (pH 6.8) and 0.1% SDS. The gels were run at 120 V constant voltage for approximately 15 h and until the bromophenol blue tracking dye was 5–10 mm from the end of the gel. The gels were stained with Coomassie blue, then destained and then sliced. The slices (0.33 cm) were dried at 60°C and then dissolved in alkaline hydrogen peroxide following the method of Goodman and Matzura [14]. Samples were counted in 4 ml of scintillant at an efficiency of about 25%.

Results

Subtilisin treatment of erythrocyte membranes labelled with cytochalasin B show two major peaks at about 15 kDa and 6–7 kDa. When the transporter is labelled at the exofacial site with ASA-BMPA and then treated with subtilisin a similar pattern results with major peaks at about 15 kDa and 6–7 kDa (Fig. 1). Extensive digestion with chymotrypsin showed breakdown of the major labelled 19 kDa fragment into smaller fragments of around 7 kDa. Cytochalasin B labelled and ASA-BMPA labelled transporter produced a parallel fragmentation pattern (Ref. 9 and present authors, unpublished results). These proteolysis results support the view that internal and external sites are very close to one another.

Ishii et al. [15] have used the glutamate specific

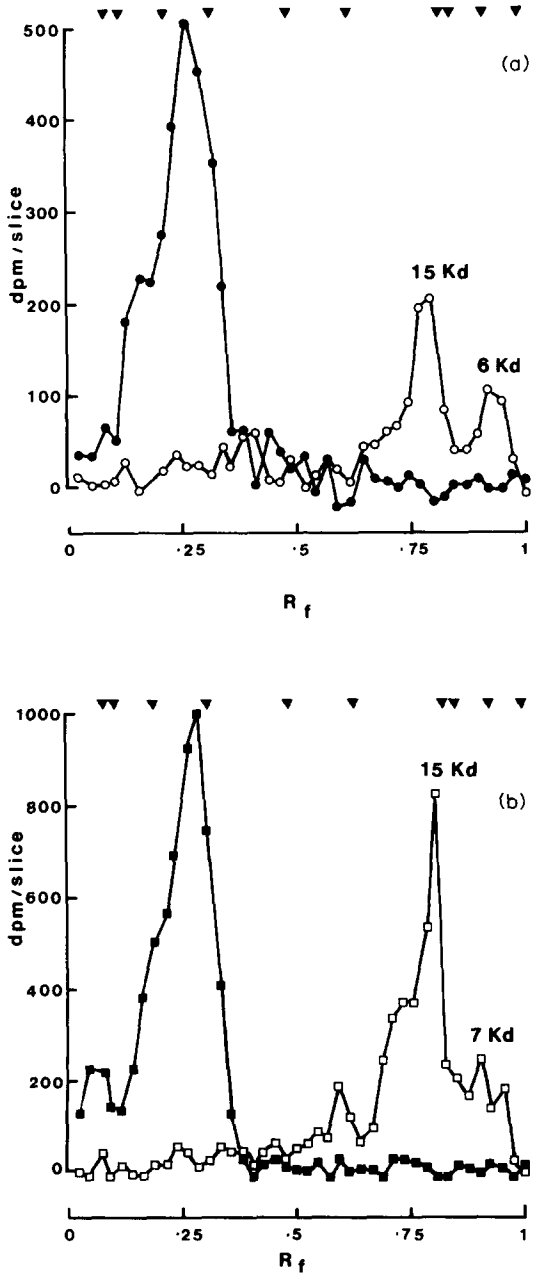

Fig. 1. Subtilisin digestion of the glucose transporter. The upper pannel (a) shows the distribution of radioactivity in membrane protein from ASA-BMPA labelled cells before (●) and after (○) digestion with 0.02 units/ml subtilisin. The lower panel (b) shows the distribution of radioactivity in membranes labelled with cytochalasin B before (■) and after (□) digestion with 0.02 units/ml subtilisin. (▼) indicates the positions of molecular weight standard proteins listed in Materials and Methods.

Staphylococcus aureus V8 proteinase to cleave the cytochalasin B labelled transporter. They found major peaks at 24 kDa and 15 kDa. We have used this proteinase on ASA-BMPA labelled transporter and find fragments of 19 kDa and 16 kDa (Fig. 2). A parallel experiment in which the transporter was labelled with cytochalasin B showed the same fragments with a slightly greater proportion of the 16 kDa fragment. The slight discrepancy between our results and those of Ishii et al. [15] may be due to differences in proteolysis conditions. Ishii et al. solubilised the purified transporter in SDS before proteolysis. The relevance of the V8 proteinase result to our present study is that it again shows parallel digestion of the externally and internally labelled transporter down to a 16 kDa fragment which is quite a small portion of the total transporter mass of 54 kDa.

Cairns et al. [6] showed that cytochalasin B labelled transporter was cleaved by NTCB into a 15.5 kDa fragment. We have confirmed this observation and also show that ASA-BMPA labelled transporter shows the same 15 kDa fragment (Fig. 3). We have found, as did Cairns et al, that the NTCB cleavage is variable and often not very extensive. This variation may be due to difficulties in maintaining the alkaline pH of the solution used to cleave the NTCB substituted residues. However, over the course of a series of numerous cytochalasin B labelling experiments we obtained evidence that the 15 kDa fragment could be further cleaved to a smaller fragment of 5.5 kDa (Fig. 4). Extensive cleavage of the ASA-BMPA labelled transporter down to a 5.5 kDa fragment was not attempted.

In order to more fully dissect the transporter we have used the tryptophan specific reagent *N*-bromosuccinimide. Funatsu et al. [16] have shown that this reagent in urea cleaves at tryptophan residues 200 times more quickly than at other amino-acid residues. It has been used in mapping the Band 3 anion transporter [17]. The pattern of fragmentation of the ASA-BMPA labelled membranes is markedly different from that of the cytochalasin B labelled membranes (Fig. 5). ASA-BMPA labelled transporter is cleaved into fragments with molecular weights ranging from 32 kDa to 19 kDa. There is little or no evidence for smaller fragments. Only the cytochalasin B labelled

membranes produce a 13 kDa and a 3 kDa fragment. (The estimate of 13 kDa for the large fragment is an average from several experiments. From Figs. 5a and 5b the estimates are 11 kDa and 14 kDa, respectively). At first we tried to reconcile these results with the suggestion that the cyto-

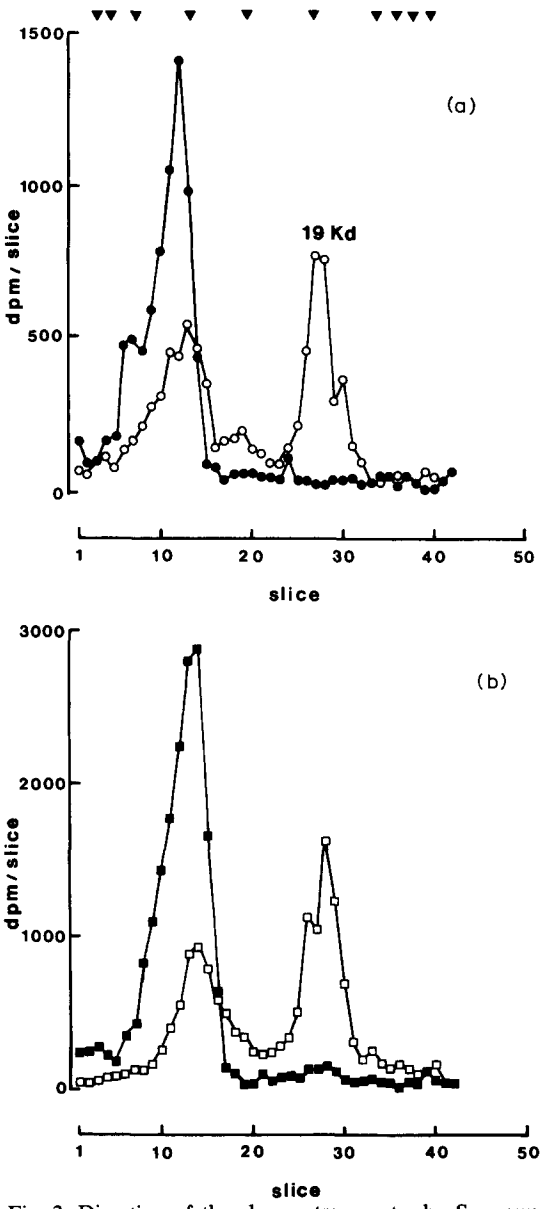

Fig. 2. Digestion of the glucose transporter by *S. aureus* V 8 proteinase. The upper panel (a) shows the distribution of radioactivity in membrane protein from ASA-PMBA labelled cells before (●) and after (○) digestion with 86 units/ml V 8 proteinase. The lower panel (b) shows the distribution of radioactivity in membranes labelled with cytochalasin B before (■) and after (□) digestion with 86 units/ml V 8 proteinase. (▼) indicates the positions of the molecular weight standard proteins listed in Materials and Methods.

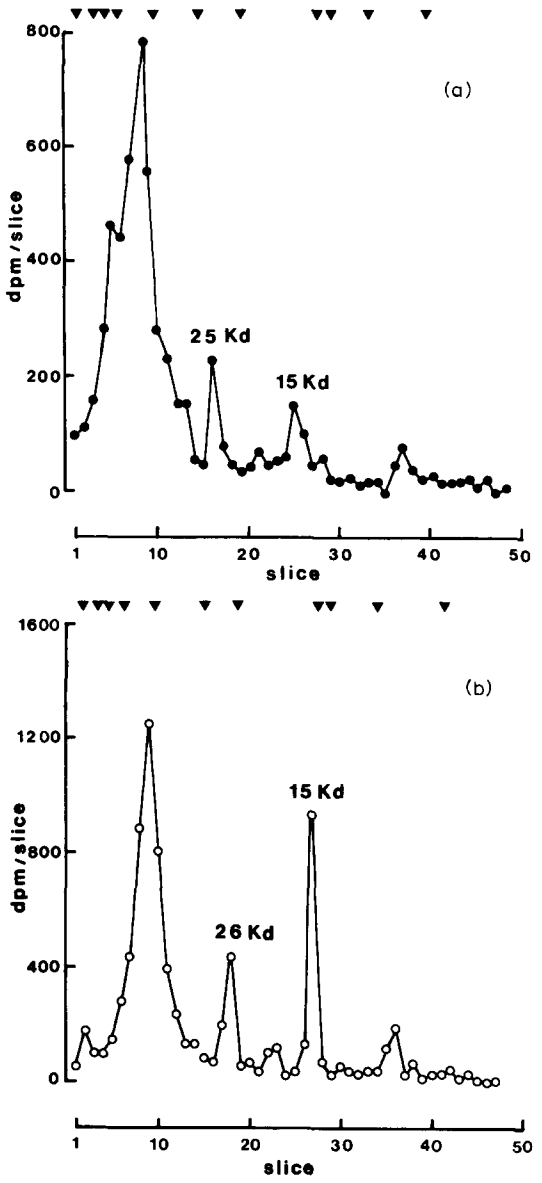

Fig. 3. Cleavage of the glucose transporter with 2-nitro-5-thiocyanobenzoic acid. Membranes were prepared from cells labelled with ASA-BMPA and a separate batch of membranes labelled with cytochalasin B. The membranes were treated with 10 mM NTCB as described in the text. The upper panel (a) shows the distribution of radioactivity in membranes labelled with ASA-BMPA (318 μg of protein was applied to the gel). The lower panel (b) shows the distribution of radioactivity in membranes labelled with cytochalasin B (316 μg of protein was applied to the gel). (▼) indicates the positions of the molecular weight standard proteins listed in Materials and Methods.

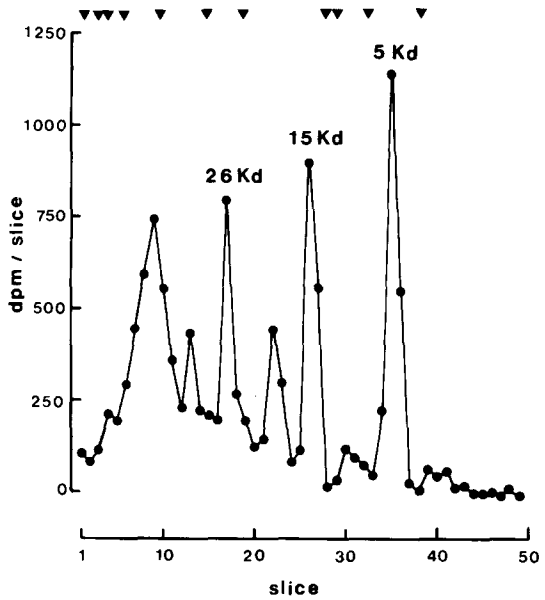

Fig. 4. More extensive cleavage of the cytochalasin B labelled glucose transporter with NTCB. The conditions of the reaction were not significantly different from those used for the experiment shown in Fig. 3. 250 μg of membrane protein was applied to the gel. (▼) indicates the positions of the molecular weight standard proteins listed in Materials and Methods.

chalasin B binding site was at the N-terminus [8]. However, we could not do so. The main problem is that 4 out of 6 of the cleavable tryptophans are in the centre of the protein or near the C-terminus. If the cytochalasin B binding site is at the N-terminus then it is not possible to assign a location for the labelled 13 kDa fragment. We have examined an alternative assignment in which the cytochalasin B site on a 11.5 kDa portion from tryptophan 388 to the C-terminus. Since a 3 kDa cytochalasin B fragment is also produced we conclude that the most likely location of the cytochalasin B binding site is between tryptophan 388 and 412 (2.7 kDa). Cleavage at tryptophan 388 must divide the cytochalasin B site from the ASA-BMPA site since ASA-BMPA labelled membranes do not give the 13 kDa fragment. Other assignments for fragments of molecular weight 32 kDa, 26 kDa, 23 kDa and 19 kDa also follow from this postulate and in Table I the theoretical and observed tryptophan fragments are shown to be in reasonable agreement. The first tryptophan cleavage is probably at tryptophan 186 since both

TABLE I

THE FRAGMENTATION OF PHOTOCHEMICALLY LABELLED HEXOSE TRANSPORTER

The apparent molecular weights of the fragments (App. frag. wt.) were estimated from calibration curves using the standard proteins shown in the figures. The assignments and assigned fragment molecular weights (Frag. wt.) are based on the transporter sequence of Mueckler et al. [1]. The ASA-BMPA site is assigned to the outside of H 9 and H 10. Photochemical labelling of residues on both sides of tryptophan 363 probably occurs. The cytochalasin B site is assigned to the inside of H 10. Some of the unassigned minor cytochalasin B fragments are probably due to slight photochemical labelling of H 9. NBS, N-bromosuccinimide; CB, cytochalasin B.

Reagent	Label	App. frag. wt. (kDa)	Assignment (residue)	Frag. wt. (kDa)
NTCB	CB and	26	207–420	24.0
	ASA-BMPA	15	347–492	16.1
		5.5	347–420	8.1
NBS	CB and	32	186–492	34.1
	ASA-BMPA	26	186–412	25.1
NBS	CB	13	388–492	11.5
		≈ 3	388–412	2.7
NBS	ASA-BMPA	23	186–388	22.6
		19	186–363	19.9
Thermo-lysin	CB	18	327–492 or 274–492	18.2 23.8

cytochalasin B and ASA-BMPA labelled membranes show a small 32 kDa fragment when treated with a very low concentration of reagent (Figs. 5 a and b).

Thermolysin treatment of ASA-BPMA labelled membranes is shown in Fig. 6. There is virtually no digestion. Coomassie blue staining showed extensive proteolysis of other erythrocyte membrane proteins. A parallel experiment in which cytochalasin B labelled membranes were treated identically with thermolysin is also shown. Coomassie blue staining of this gel showed an identical overall pattern of general proteolysis. Clearly the cytochalasin B labelled transporter is much more susceptible to thermolysin cleavage than is the ASA-BMPA labelled transporter. There is a single thermolysin fragment in cytochalasin B labelled membranes with an estimated molecular weight of 18 kDa. This result is in marked contrast to that obtained by Shanahan and D'Artel-Ellis [8] who

suggested that thermolysin cleaved a very small labelled fragment from the N-terminus which resulted in a total loss of cytochalasin B label but with no appearance of a large fragment. We do not fully understand the reason for this discrepancy. We have repeated the experiment four

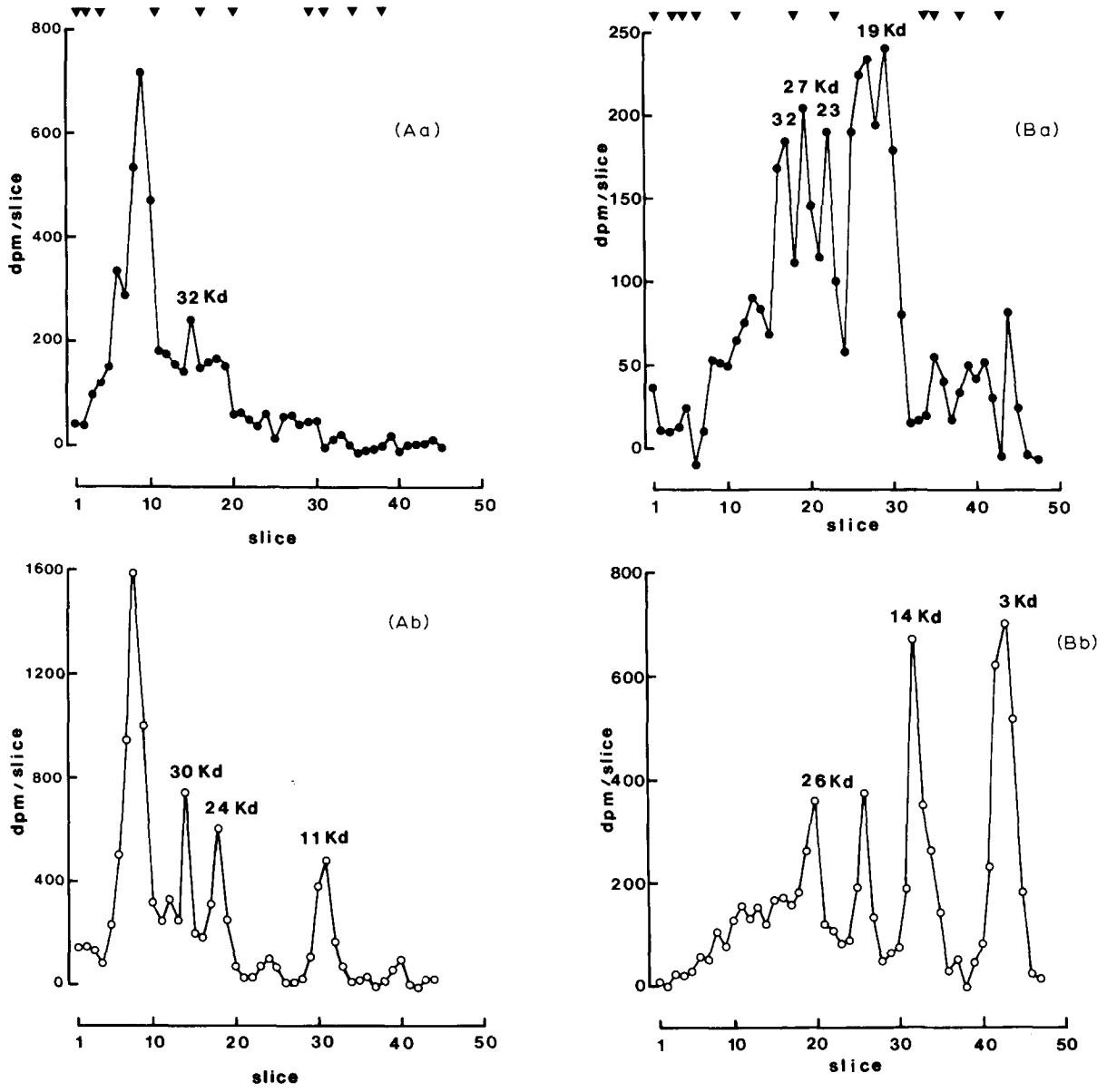

Fig. 5. Cleavage of the glucose transporter with *N*-bromosuccinimide. Panels A show the result of treatment with 0.05 μg NBS/μg of membrane protein. The upper pannel (Aa) shows the distribution of radioactivity from ASA-BMPA labelled membranes. The lower panel (Ab) shows the distribution of radioactivity in membranes labelled with cytochalasin B. Panels B show the result of treatment with 0.5 μg NBS/μg of membrane protein. The upper panel (Ba) shows the distribution of radioactivity from membranes labelled with ASA-BMPA. The lower panel (Bb) shows the distribution of radioactivity in membranes labelled with cytochalasin B. (▼) indicates the position of the molecular weight standard proteins listed in Materials and Methods.

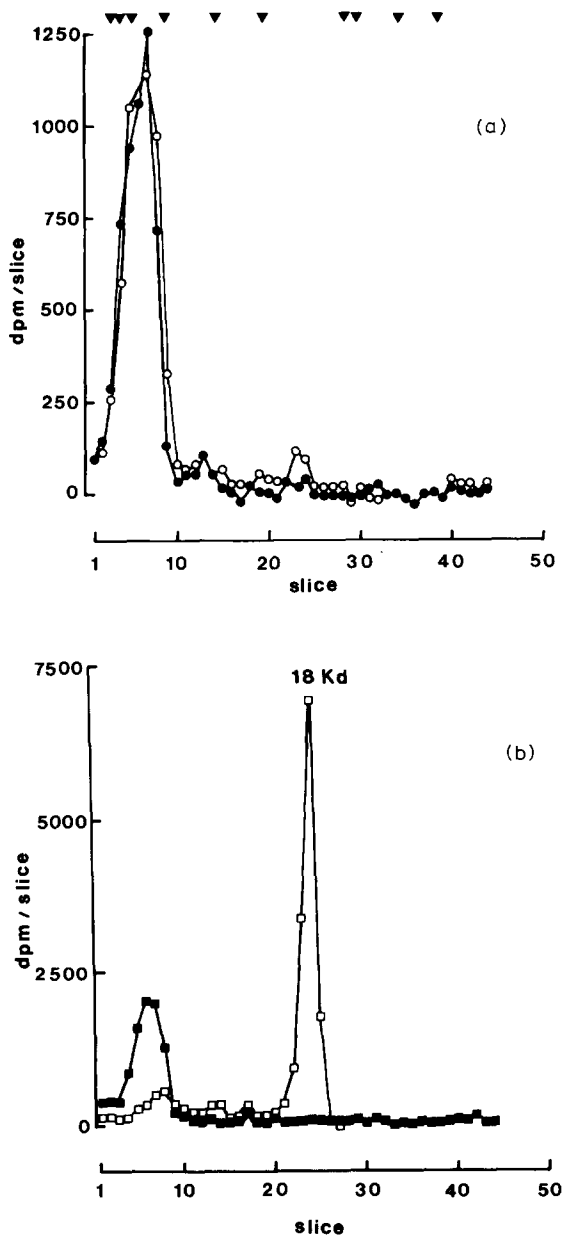

Fig. 6. The effect of thermolysin digestion of the glucose transporter. Membranes were prepared labelled with either ASA-BMPA or with cytochalasin B. One half of each batch was digested with 0.4 units/ml thermolysin for 30 min at 20 °C. The upper panel (a) shows the distribution of radioactivity in ASA-BMPA labelled membranes before (●) and after (○) thermolysin digestion (325 μg of membrane protein in each track). The lower panel (b) shows the distribution of radioactivity in membranes labelled with cytochalasin B before (■) and after (□) thermolysin digestion (212 μg of membrane protein in each track). (▼) indicates the positions of the molecular weight standard proteins listed in Materials and Methods.

times with two different batches of thermolysin but always with the same result. After thermolysin treatment the membranes become slightly more difficult to sediment and therefore in some cases we have corrected for an overall loss of protein by adjusting the amount of control protein added to the gel. However, in most experiments (and including the experiment shown in Fig. 6) this was unnecessary as the recovery of proteolysed membranes was usually high. It is not clear from the data of Shanahan and D'Artel-Ellis whether the possibility of poor recovery of proteolysed membrane protein has been taken into account. We can, however, confirm that thermolysin probably cleaves at the inside of the transporter since there is virtually no thermolysin cleavage of the transporter in resealed ghosts (data not shown).

The thermolysin result we have obtained is indicative of a major conformational difference between transporter which has ligand bound at either the internal or external sites. When ASA-BMPA is bound externally there must be a large shape change which reduces the availability of an internal thermolysin cleavage site.

Discussion

Sequence analysis of the labelled hexose transporter fragments will be required before a definitive statement can be made about the location of the binding sites. Nevertheless a tentative model for the possible locations of the binding sites aids the discussion of the observed fragmentation patterns. Fig. 7 shows the postulated location of the internal cytochalasin B site and the external ASA-BPMA site on the hexose transporter.

We have adopted these assignments mainly because of the results with NTCB and with *N*-bromosuccinimide. We propose that the *N*-bromosuccinimide cleaves initially at tryptophan 186 to give a 32 kDa (34 kDa theoretical) fragment containing both the internal and external sites. Another important tryptophan cleavage site occurs at tryptophan 388 which gives a 13 kDa fragment (11.5 kDa theoretical) containing the cytochalasin B site but not the ASA-BMPA site. Further cleavage of this then gives a labelled 3 kDa fragment plus a C-terminal unlabelled fragment. Thus the cytochalasin B site probably lies

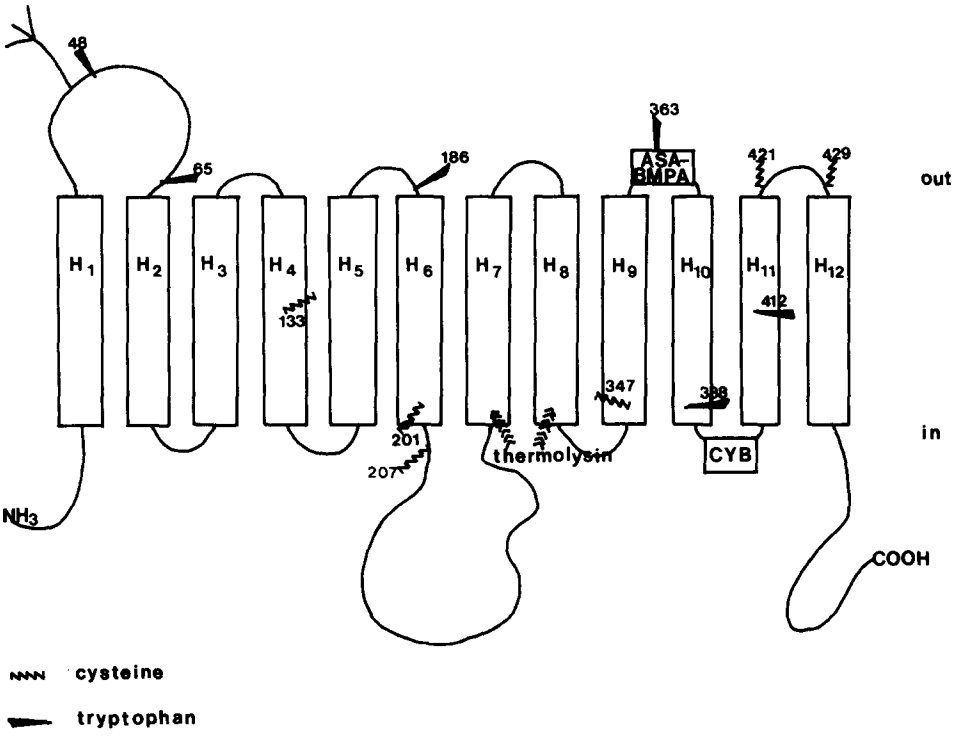

Fig. 7. The proposed locations of the ASA-BMPA and cytochalasin B sites on the glucose transporter. The assignments and diagram are based on the transporter sequence given by Mueckler et al. [1]. The positions of the cleavable tryptophans and cysteines are indicated. Also shown is the proposed thermolysin cleavage site. It is proposed that the core of the transporter (including internal and external sites plus a possible hydrogen bonding channel) is located in membrane spanning segments H 7–H 10.

between hydrophobic spans H 10 and H 11 with the ASA-BMPA site distal to tryptophan 388. ASA-BMPA labelled transporter has a peak in common with cytochalasin B labelled transporter at 26 kDa (possibly residues 186–412). ASA-BMPA shows an additional peak at 23 kDa which would be consistent with the loss of the 3 kDa cytochalasin B site from the common 26 kDa fragment. Because there are possible inaccuracies in fragment molecular weight estimations further speculation about the locations of some minor cytochalasin B fragments is rather uncertain. We assign the ASA-BMPA site to the outer surface of H 9 because proteolysis with subtilisin and with chymotrypsin showed parallel production of a 7 kDa fragment. Thus although the ASA-BMPA site is more distal than tryptophan 388 it cannot be more than 1 or 2 spans away from the cytochalasin B site but has to be located within the 15 kDa NTCB fragment (H 9-H 12, see below).

The proposed location of the active sites around H 9, H 10 is consistent with both sites being located on the trypsin fragment between residues 257 and 456 [25]. Cairns et al. [6] have shown that a 15.5 kDa cytochalasin B labelled fragment is produced by the thiol reagent NTCB. This leaves an unlabelled 38 kDa fragment. The results of Cairns et al. [6,25] are consistent with an assignments for the cytochalasin B site around H 10. Thus if the major thiol cleavage site is at cysteine 347 then this would give an unlabelled 38 kDa fragment (residues 1–347) plus a 16 kDa fragment containing the cytochalasin B binding site from the remaining C-terminus. The 38 kDa fragment would include the carbohydrate attachment site at asparagine 45. In the publication of Cairns et al. [6] endoglycosidase is shown to sharpen the 38 kDa fragment which would be consistent with loss of carbohydrate. If the assignment of the cysteine cleavage at residue 347 is correct then it would be

predicted that the 15 kDa (16.1 kDa theoretical) NTCB fragment should be further cleavable down to a smaller labelled 8 kDa fragment (cysteines 347–421). Using cytochalasin B labelled transporter we were able to show cleavage of the 15 kDa fragment to a labelled 5.5 kDa fragment. The theoretical 8 kDa fragment may be so hydrophobic that it runs anomalously when compared with soluble molecular weight standard proteins. The importance of the observation that the 15 kDa fragment is further cleavable is that it can no longer be easily assigned to the N-terminus (residues 1–133) since this N-terminal cysteine fragment contains no additional cysteine residues and thus should not be further fragmented.

Thermolysin gives a completely different result when transporter is labelled with ASA-BMPA compared with transporter labelled with cytochalasin B. ASA-BMPA virtually completely protects the transporter from thermolysin cleavage while cytochalasin B labelled transporter is rapidly cleaved into a 18 kDa fragment. Although thermolysin has broad specificity it shows some selectivity for leucine, isoleucine, phenylalanine and valine residues [18]. In some cases thermolysin has been used for detecting changes in protein conformation [18]. Part of the specificity of the thermolysin cleavage of the transporter may be related to the low enzyme concentration used. The use of higher enzyme concentrations resulted in poor recovery of membrane proteins but a doubling of the thermolysin concentration still did not result in cleavage of the ASA-BMPA labelled transporter (data not shown). If a double cleavage occurs, as is the case with trypsin, then the conformation sensitive thermolysin cleavage site could be assigned to the hydrophilic internal loop region or to the 4 kDa cytosolic portion of the C-terminus. However, a single cleavage seems equally likely at present. The fragment molecular weight is about 18 kDa which could correspond to cleavage in the region of the Ile-Leu-Ile-Ala-Val-Val sequence at the cytosolic side of H 7 or the Leu-Phe-Val-Val sequence at the cytosolic side of H 8. As pointed out by Mueckler et al. [1] H 7 and H 8 contain many serine and glutamine residues that could form a hydrogen bonding channel through which transported sugar could move. Thus the region is likely to be sensitive to a conformational change which, on ASA-BMPA binding, moves the thermolysin cleavage site into a more hindered membrane domain. This external ligand induced closure of the inside of a possible transport channel is mechanistically very interesting and is consistent with the postulate of many workers [18–21] that the inside of the transporter is not available when ligand is bound outside. Barnett et al. [22] also suggested differences in the conformational state of the transporter when sugar analogues bind to either the inside or the outside of the transporter. It remains to be established whether there are significant conformational changes in the transporter in the absence of substrate. This is of course the central tenet of the carrier model for hexose transport [23].

The assignment of a hydrogen bonding transport channel in membrane spanning segments H 7 and H 8 is also consistent with ASA-BMPA labelling of H 9 if protein folding is taken into account. Barnett et al. [24] suggested from sugar specificity investigations that a hydrophobic cleft was adjacent to a more hydrophilic region of the transporter and that the latter was involved in hydrogen bonding of sugar hydroxyls. The adjacent hydrophobic region was considered to be responsible for the enhanced interaction of sugar analogues which were substituted with hydrophobic groups at C-4 and C-6. Thus the azidosalicoyl group is likely to interact with and consequently label the adjacent hydrophobic residues (H 9) rather than the hydrogen bonding residues in the putative channel (H 7–H 8).

The speculations concerning active-site location that we have described should aid the design of additional experimental tests for the assignments. Sequence analysis of the labelled peptide fragments could be used to test the suggestions made here but such a study could be complicated by the instability of the photoprobes during acid hydrolysis of the peptide bonds.

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