Biochem. J. (1988) 256, 421–427 (Printed in Great Britain)

Proteolytic dissection as a probe of conformational changes in the human erythrocyte glucose transport protein

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Tryptic digestion has been used to investigate the conformational changes associated with substrate translocation by the human erythrocyte glucose transporter. The effects of substrates and inhibitors of transport on the rates of tryptic cleavage at the cytoplasmic surface of the membrane have confirmed previous observations that this protein can adopt at least two conformations. In the presence of phloretin or 4,6-*O*-ethylidene-D-glucose, the rate of cleavage is slowed. Because these inhibitors bind preferentially at the extracellular surface of the transporter, their effects must result from a conformational change rather than from steric hindrance. A conformational change must also be responsible for the effect of the physiological substrate D-glucose, which is to increase the rate of cleavage. The regions of the protein involved in the conformational changes include both of the large cytoplasmic regions that are cleaved by trypsin: these are the central hydrophilic region of the sequence (residues 213–269) and the hydrophilic *C*-terminal region (residues 457–492).

INTRODUCTION

The amino acid sequence of the human erythrocyte membrane glucose transporter has recently been deduced from the nucleotide sequence of a cDNA clone encoding an apparently identical protein from HepG2 hepatoma cells [1]. The protein is known to be predominantly α-helical [2–6], and hydropathic analysis of the sequence suggests that it contains 12 hydrophobic, membrane-spanning α-helical segments [1]. Several of these putative membrane-spanning helices are predicted to be amphipathic and may be clustered around an aqueous channel through which the glucose moves [1,6,7]. The major hydrophilic regions of the sequence are proposed to be exposed on the cytoplasmic surface of the membrane. These include the *N*-terminal region (residues 1–12), the *C*-terminal region (residues 451–492) and a large extramembranous loop near the centre of the sequence (residues 207–271) [1]. The cytoplasmic locations of the latter two regions have in fact recently been confirmed both by vectorial tryptic-digestion experiments [1,8–10], and by the use of peptide-specific antibodies [11].

Despite these considerable recent advances in our understanding of the transporter structure, the mechanism of transport remains unclear. Two main types of kinetic model have been proposed to account for the observed features of the transport process. In the alternating conformation model, a single substrate-binding site is alternately exposed at the two surfaces of the membrane by means of a conformational change [12–14]. In contrast, a recent model proposed by Carruthers and colleagues on the basis of fluorescence quenching [15,16] and ligand binding [17] studies, suggests that two substrate-binding sites are simultaneously present, one exposed at each face of the membrane. Although the two models are fundamentally different in many respects, both imply that conformational changes in the transport protein are central to the molecular mechanism of transport. Such changes have in fact been detected by a large variety of techniques [5,6,18–23].

Although evidence for the involvement of conformational changes in the mechanism of transport is strong, the identity of those regions of the protein that are affected by the changes remains largely unknown. A recent report on the inactivation of transport by alkylating agents suggested that substrate-induced conformational changes occur primarily within the hydrophobic, membrane-spanning regions of the protein, while the hydrophilic segments outside the bilayer are largely unaffected [24]. In the present study, we have used tryptic digestion to investigate more fully the possible involvement of the hydrophilic, cytoplasmic domain of the protein in transport-related conformational changes. Trypsin cleaves the transporter, in the absence of substrates or inhibitors of transport, solely at the cytoplasmic surface of the membrane [8,9]. Cleavage occurs at several sites in the central extramembranous loop of the sequence between Arg-212 and Arg-269, and in the *C*-terminal region of the sequence, following Lys-456 [10]. After short periods of digestion, cleavage within the central loop yields a glycosylated fragment, derived from the *N*-terminal half of the protein, which runs as a broad band of apparent M_r 23000–42000 on SDS/polyacrylamide gels [8,11,25]. The *C*-terminal half of the protein initially yields a tryptic fragment which migrates as a sharp band of apparent M_r 25500. The latter is subsequently converted via a fragment of apparent M_r 23000 to a stable product of apparent M_r 18000, which lacks the *C*-terminus of the intact protein [8,11,25]. However, the order in which the cleavages occur appears not to be obligatory; cleavage of the intact transporter can occur first near its *C*-terminus, yielding substantial amounts of a glycosylated fragment of apparent M_r 45000 after short periods of digestion [11].

We describe in this report the effects of D-glucose and of reversible inhibitors of transport on the rate and

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pattern of these tryptic cleavages of the transporter. The inhibitors used were phenyl β-D-glucoside and cytochalasin B, which bind preferentially at the cytoplasmic side of the membrane [13,26], and 4,6-*O*-ethylidene-D-glucose and phloretin, which bind preferentially at the extracellular side of the membrane [27,28]. These inhibitors would be predicted by the alternating conformation model for transport to bind preferentially to the inward-facing and outward-facing conformations of the protein respectively, and so cause it to accumulate in one of these forms. The resultant effects on the cleavage of the transporter by trypsin have, for the first time, identified specific regions of the protein sequence that are involved in conformational changes.

A preliminary account of part of this work was presented at the 623rd meeting of The Biochemical Society in 1987 [28a].

EXPERIMENTAL

Materials

Cytochalasin B and 4,6-*O*-ethylidene-α-D-glucose were obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Bovine lung aprotinin was from the Boehringer Corporation (Lewes, East Sussex, U.K.). Phloretin, phenyl β-D-glucopyranoside, L-glucose, D-glucose, diphenylcarbamyl chloride-treated trypsin and molecular-mass markers for SDS/polyacrylamide-gel electrophoresis were supplied by Sigma (Poole, Dorset, U.K.). New England Nuclear (Southampton, Hants., U.K.) supplied [4-^{3}H]cytochalasin B. Outdated human blood was provided by the blood bank of the Royal Free Hospital.

Preparation of the human erythrocyte glucose transporter

The human erythrocyte glucose transporter was prepared from outdated blood by the method of Baldwin *et al.* [29], with the modifications described by Cairns *et al.* [25]. The cytochalasin B-binding activity of each preparation was measured by equilibrium dialysis as previously described [30]. Only those preparations that contained more than 11 nmol of cytochalasin B-binding sites per mg of protein were used in this study. All showed a single broad band of Coomassie Blue-staining material of average apparent M_r 55000 on SDS/polyacrylamide-gel electrophoresis.

Tryptic digestion

Tryptic digestion was performed in 50 mM-sodium phosphate/100 mM-NaCl/1 mM-EDTA, pH 7.4, in the presence or absence of sugars or transport inhibitors. The respective concentrations of purified transporter and of trypsin were 216 μg/ml and 2.5 μg/ml unless stated otherwise. At various times during the digestions, samples were taken for analysis by SDS/polyacrylamide-gel electrophoresis. The trypsin present in these samples was immediately inactivated by the addition of a 2-fold excess (by weight) of bovine lung aprotinin.

Control experiments were carried out using alkali-stripped human erythrocyte membranes [29] to ensure that any effects of substrates and inhibitors of glucose transport on the rate of transporter cleavage resulted from their binding to the transporter rather than to trypsin. None of the compounds tested had any effect on the rate of tryptic cleavage of the anion transporter in these membranes under conditions identical with those used for digestion of the purified glucose transporter (results not shown). However, their effects on the rate of production of tryptic fragments of the glucose transporter in these membranes were similar to those seen using the purified protein (results not shown).

The apparent rate of cleavage of the protein by trypsin varied slightly between transporter preparations, probably because of their variable content of sealed, vesicular material in which the cytoplasmic domain of the transporter is not accessible for cleavage. Therefore, comparisons of the effects of different sugars or inhibitors on the cleavage rate were always made by performing parallel digestion experiments simultaneously, using identical trypsin and transporter preparations. The proportion of transporters inaccessible to trypsin was estimated from the amount of transporter remaining uncleaved after extended periods of digestion. Typically, about 25% of the transporter remained intact even after treatment with protease for 26 h at 25 °C, fresh trypsin (5%, w/w) being added after 0, 3 and 6 h.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out by the procedure of Laemmli [31] using 12% polyacrylamide gels. Proteins of known M_r used as markers were as previously described [25]. After fixing and staining with Coomassie Blue the gels were scanned spectrophotometrically at 530 nm. Previous studies (D. A. Elliot & S. A. Baldwin, unpublished work) have shown a linear relationship between peak area and the amount of transporter on a gel over the concentration range used here. Routinely, the peak area data were corrected for the presence of transporter inaccessible to trypsin by subtracting the value for intact transporter remaining after extended trypsin treatment, as described above. After correction in this fashion the data indicated the percentage of the 'accessible' transporters remaining intact after various periods of tryptic digestion. They were then fitted to straight-line, first-order plots by linear regression.

RESULTS

The rate of cleavage of the membrane-bound glucose transporter by trypsin at 25 °C, in the presence of the non-transported substrate analogue 4,6-*O*-ethylidene-D-glucose (ethylidene glucose) at a concentration of 200 mM, was found to be markedly lower than in the presence of 200 mM-L-glucose, which does not bind to the protein (Figs. 1 and 2). The extents of cleavage were measured by spectrophotometric scanning of the gels shown in Fig. 1. This procedure slightly underestimates the true extent of cleavage because the M_r 45000 tryptic fragment (arrowed in Fig. 1) is not fully resolved from the broad M_r 55000 band of the intact transporter. However, after correction for the presence of 28% of the transporters that were inaccessible to trypsin, the cleavage data showed apparent first-order kinetics (Fig. 2). Ethylidene glucose was found to decrease the first-order rate constant for cleavage by about 4-fold relative to L-glucose (Table 1). Inspection of the gels showed that the patterns of fragments produced in the presence of the

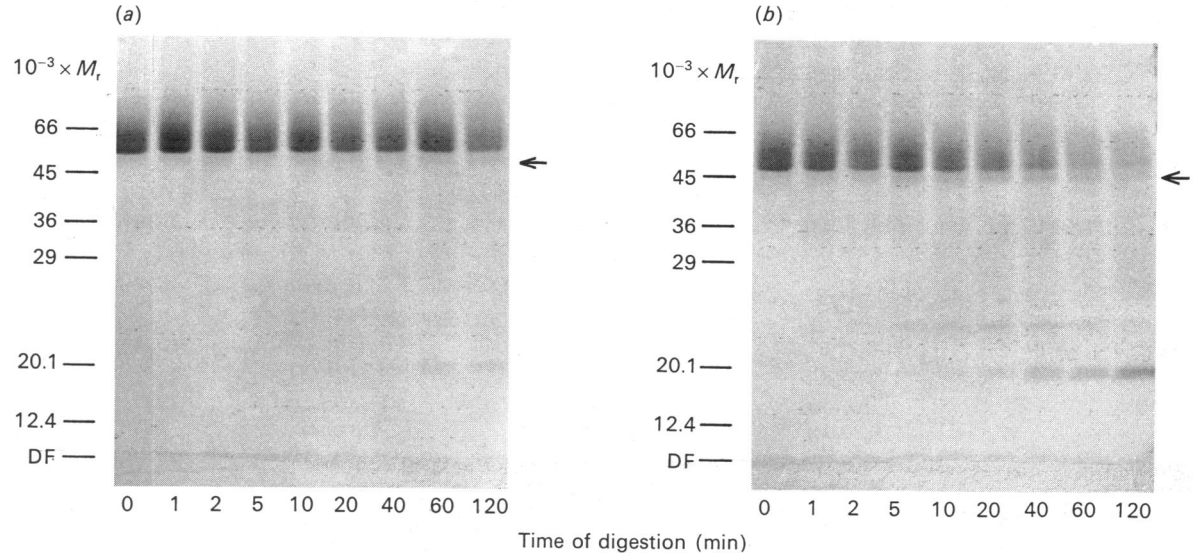

Fig. 1. The effect of ethylidene glucose on the tryptic cleavage of the glucose transporter

Glucose transporter (216 μg/ml) was digested at 25 °C with 1.2% (w/w) trypsin for the times indicated, in the presence of either (*a*) 200 mM-ethylidene glucose or (*b*) 200 mM-L-glucose. Samples were electrophoresed on a 12% SDS/polyacrylamide gel and then stained with Coomassie Blue. The positions of marker proteins of known M_r, and of the glycosylated M_r 45000 fragment of the transporter (arrowed), are indicated. DF = tracking dye front.

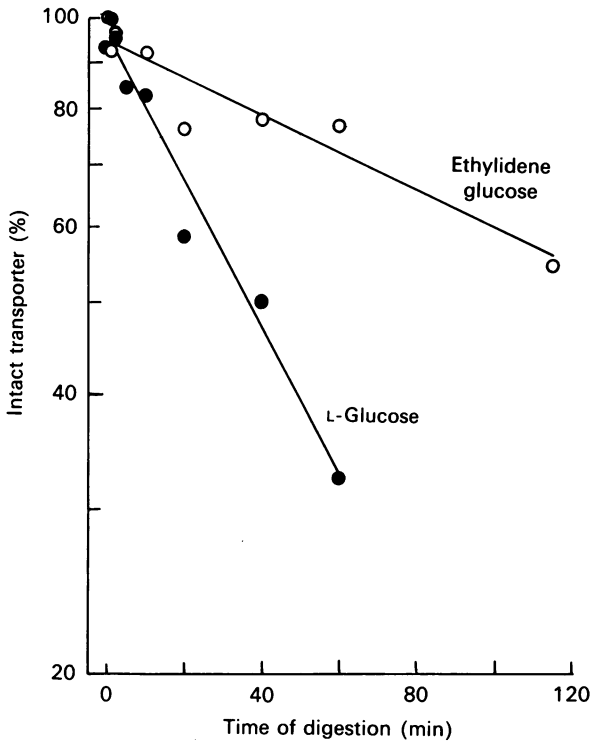

Fig. 2. The effect of ethylidene glucose on the rate of tryptic cleavage of the glucose transporter

The relative amounts of intact transporter remaining after various times of tryptic digestion in the presence of 200 mM-ethylidene glucose (○) or 200 mM-L-glucose (●) were determined from spectrophotometric scans of the gels shown in Fig. 1. The data are shown as a semi-logarithmic plot after correction for the presence of transporters inaccessible to trypsin as described in the Experimental section. The straight lines have been fitted by linear regression.

two sugars were the same, but that the rates of production both of the M_r 45000 fragment (arrowed in Fig. 1) and of the M_r 18000 fragment and its precursors were reduced in the presence of ethylidene glucose. This finding was confirmed by spectrophotometric scanning to quantify the M_r 18000 fragment (results not shown).

The mechanism of the different effects of L-glucose and ethylidene glucose was further investigated by examining the effects of different concentrations of the two sugars on tryptic cleavage. In this experiment the transporter (160 μg/ml) was incubated for a fixed period (4 h) with trypsin (1%, w/w) at 25 °C, and the effects of the sugars were monitored by quantifying the amounts of both the uncleaved transporter and its M_r 18000 tryptic fragment on a gel. The extent of cleavage was little affected by L-glucose at concentrations up to 400 mM, but decreased as the concentration of ethylidene glucose increased up to 400 mM (Fig. 3). The half-maximal effect of the sugar on the amount of the M_r 18000 fragment produced was seen at about 25 mM-ethylidene glucose. The concentration required for half-maximal effect on the amount of the intact transporter remaining after digestion appeared to be higher, at approximately 75 mM. However, less confidence can be placed upon this estimate because the presence of the M_r 45000 tryptic fragment precludes accurate quantification of the intact transporter, as mentioned above. Accurate estimation of the M_r 18000 fragment is more easily achieved because it is well resolved from other fragments on the gel.

The effects of phloretin upon the rate of tryptic cleavage of the transporter were also investigated. This compound is a potent inhibitor of transport and resembles ethylidene glucose in that it binds at the extracellular surface of the transporter [28]. The effect of phloretin (100 μM) was similar to that of ethylidene glucose; the rate of cleavage of the transporter by trypsin was slowed and this effect was manifested as a decrease in the rate of production of both the M_r 45000 and the M_r 18000 tryptic fragments

Table 1. The effects of substrate and inhibitors on the rate of tryptic cleavage of the glucose transporter

Rate constants were calculated from the tryptic cleavage data after correction for the presence of inaccessible transporters, as described in the Experimental section. Each value, and its associated S.E.M., was calculated from the results of a single experiment using linear regression analysis.

Temperature (°C)	Inhibitor/substrate	$10^3 \times$ Rate constant (min^{-1})	Control	$10^3 \times$ Rate constant (min^{-1})
25	Ethylidene glucose (200 mM)	4.5±0.6	L-Glucose (200 mM)	17.9±1.2
	Phloretin (100 μM)	5.5±0.4	Ethanol (1%, v/v)	15.9±0.8
	D-Glucose (200 mM)	21.6±1.6	L-Glucose (200 mM)	13.6±0.8
	D-Glucose (200 mM)	19.8±1.4	L-Glucose (200 mM)	12.8±0.7
45	Ethylidene glucose (200 mM)	9.6±2.1	L-Glucose (200 mM)	37.0±2.3
	Phloretin (100 μM)	16.3±1.5	Ethanol (1%, v/v)	36.2±2.4
	Cytochalasin B (20 μM)	26.6±1.7	Ethanol (0.5%, v/v)	36.2±2.4
50	Cytochalasin B (20 μM)	50.0±7.0	Ethanol (0.5%, v/v)	201.0±7.0

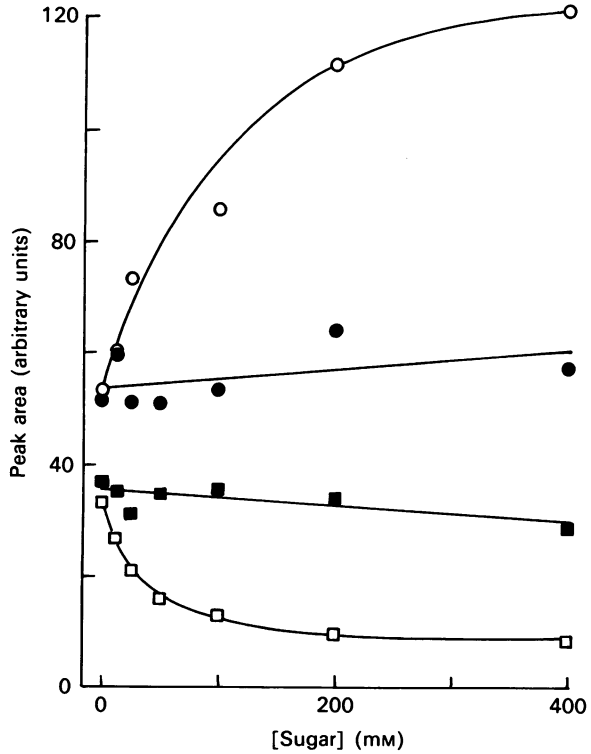

Fig. 3. The concentration dependence of the effect of ethylidene glucose on tryptic cleavage of the glucose transporter

Glucose transporter (160 μg/ml) was digested at 25 °C for 4 h with 1% (w/w) trypsin in the presence of various concentrations of L-glucose (●,■) or ethylidene glucose (○,□). Samples were electrophoresed on a 12% SDS/polyacrylamide gel and then stained with Coomassie Blue. The relative amounts of the intact transporter (●,○) and of the tryptic fragment of apparent M_r 18000 (■,□) were estimated by spectrophotometric scanning.

(results not shown). After correction for the presence of 36% of the transporter which was inaccessible to trypsin, the cleavage data exhibited first-order kinetics (Fig. 4). Phloretin decreased the first-order rate constant for cleavage about 3-fold (Table 1).

Ethylidene glucose and phloretin bind preferentially to the transporter at the extracellular surface of the membrane [27,28]. For comparison, the effects of cytochalasin B and phenyl β-D-glucoside (phenyl glucoside) on the rate of cleavage of the transporter by trypsin were investigated. These inhibitors bind preferentially at the cytoplasmic side of the membrane [13,26]. Cytochalasin B (20 μM) had no effect on the rate of tryptic cleavage of the transporter at 25 °C (results not shown). Similarly, the presence of 50 mM-phenyl glucoside slowed the rate of cleavage of the transporter, relative to a control, to a much smaller extent than either ethylidene glucose or phloretin (results not shown).

A possible explanation for the protection against tryptic cleavage afforded by ethylidene glucose and phloretin might be that these agents stabilize the transporter in a trypsin-resistant, outward-facing conformation. Conversely, if the alternating conformation model for transport is correct, cytochalasin B and phenyl glucoside might be expected to increase the rate of cleavage by stabilizing an inward-facing conformation of the transporter. No such effect was seen at 25 °C. However, analysis of the kinetics of transport by Lowe & Walmsley [32], using such an alternating conformation model, led to the prediction that the proportion of the transporter in each conformation is temperature dependent. In the human erythrocyte, at 25 °C, 75% of the transporters are predicted to be in the inward-facing conformation. Therefore, at this temperature any effects of cytochalasin B and phenyl glucoside mediated by conformational changes might be expected to be smaller than those produced by stabilizers of the outward-facing

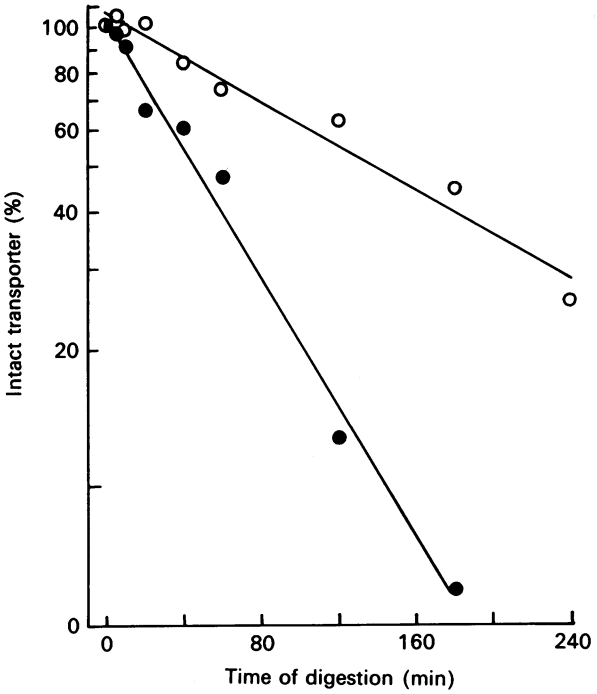

Fig. 4. The effect of phloretin on the rate of tryptic cleavage of the glucose transporter

Glucose transporter (216 μg/ml) was digested at 25 °C with 1.2% (w/w) trypsin for the times indicated, in the presence of either 100 μM-phloretin and 1% (v/v) ethanol (○), or 1% (v/v) ethanol alone (●). Samples were electrophoresed on a 12% SDS/polyacrylamide gel, stained with Coomassie Blue, and then the relative amounts of intact transporter remaining determined by spectrophotometric scanning. The data are shown as a semi-logarithmic plot after correction for the presence of transporters inaccessible to trypsin. The straight lines have been fitted by linear regression.

conformation, such as phloretin and ethylidene glucose. To investigate this possibility, digestion experiments were performed at 45 °C, at which temperature about 50% of the transporters in the erythrocyte are predicted to be in the inward-facing conformation and about 50% in the outward-facing conformation [32].

The protective effects of phloretin and of ethylidene glucose at 45 °C closely resembled those seen at 25 °C (Table 1). Phenyl glucoside (50 mM) had no significant effect on the rate of tryptic cleavage of the transporter either at 45 °C or at 50 °C (results not shown). However, cytochalasin B exerted protective effects at both temperatures; the rate constants for tryptic cleavage were decreased about 1.4-fold and 4-fold in the presence of 20 μM-cytochalasin B at 45 °C and 50 °C respectively (Table 1).

The effects of the physiological substrate, D-glucose, on tryptic cleavage of the transporter were also examined, at both 25 °C and 45 °C. At 25 °C, D-glucose (200 mM) increased the first-order rate constant for cleavage of the transporter by trypsin (1%, w/w) about 1.5-fold, relative to that seen in the presence of 200 mM-L-glucose (Table 1). Although the effect was small, it was reproducible (Table 1). However, no significant effects of D-glucose were seen at 45 °C (results not shown).

DISCUSSION

Both phloretin and ethylidene glucose were found to decrease the rate of tryptic cleavage of the purified glucose transporter at 25 °C and at 45 °C. Phloretin has been reported to bind exclusively to a site on the transporter that is exposed to the extracellular environment [17,28], whereas trypsin cleaves the protein solely at sites on the cytoplasmic surface of the membrane [8,9]. The effect of phloretin cannot, therefore, be the result of simple steric hindrance following its occupation of the binding site. Instead, it must involve a conformational change to a form of the protein in which the sites of cleavage are less accessible to trypsin.

The same explanation probably also accounts for the effect of ethylidine glucose, although this sugar analogue does not exhibit absolute specificity for the extracellular substrate-binding site [17,27]. The concentration dependence of the effect revealed in Fig. 3 indicates that it does not merely reflect a non-specific perturbation of the lipid environment of the protein. The inevitable presence of a proportion of inactive transporters in the preparations used in this study [29] complicates the kinetic analysis of the cleavage process. However, the concentration of ethylidene glucose required to afford half-maximal protection against cleavage, 25 mM, corresponds closely to the value of 26 mM measured as the K_i for inhibition of cytochalasin B binding to protein-depleted erythrocyte membranes [33]. It has been suggested that such a high K_i value reflects binding to a low-affinity site at the cytoplasmic surface of the membrane [17]. However, even if a portion of the ethylidene glucose is binding to such a site, it is unlikely for two reasons that the resultant steric hindrance is responsible for the effect on cleavage. Firstly, at the concentration where the half-maximal effect is seen (25 mM), the two-sites model predicts that only 17% of the intracellular sites would be occupied (calculated from the data in ref. [17]). Secondly, occupation of presumably the same intracellular sites by the bulky molecules phenyl glucoside and cytochalasin B causes little or no steric hindrance respectively to the action of trypsin at 25 °C.

The observation that neither cytochalasin B nor phenyl glucoside increased the rate of tryptic cleavage of the transporter even at 50 °C is at first sight difficult to reconcile with a single-site, alternating conformation model for transport in which the outward-facing conformation of the protein is more resistant to tryptic cleavage. This model has been supported by a recent series of steady-state kinetic studies [32,34,35]. A possible explanation for the results would be that the purified transporter adopts largely an inward-facing conformation even at high temperatures. The lipid environment of the purified protein is known to be different from that which it experiences in the intact erythrocyte membrane, and so the temperature dependence of its conformational states may differ from that which occurs in the cell. This altered environment may also be responsible for the finding that the transport activity of the purified transporter is much lower than that seen in the intact cell [36,37]. Alternatively, the single-site model may not adequately describe the mechanism of transport, and a more complex model, such as that of Helgerson & Carruthers [17], may be required.

Conformational changes in the protein resulting from the binding of cytochalasin B have been detected by

other techniques. For example, the binding of this inhibitor to the cytoplasmic side of the membrane protects an exofacial thiol group from reaction with maleimides [38–40]. Karim *et al.* [41] have reported that photolabelling the transporter with cytochalasin B greatly increases its susceptibility to cleavage by thermolysin. However, it was not reported whether non-covalently bound cytochalasin B also has this effect. Kurokawa *et al.* [42] have found that the covalent, but not the non-covalent, binding of cytochalasin B to the transporter leads to the increased exposure of an anionic domain and thus an alteration in chromatographic behaviour of the protein. Similarly, transporter photolabelled at its extracellular surface with an azidosalicoyl derivative of bis-mannose has been reported to be resistant to thermolytic cleavage, whereas protein labelled with cytochalasin B is readily cleaved [43]. However, it is not clear whether the noncovalent association of these two ligands with the protein has any effect on the rate of thermolytic cleavage.

The protection against tryptic cleavage of the transporter afforded by cytochalasin B at 45 °C and 50 °C may result from its ability to prevent or slow the denaturation of the protein at this elevated temperature. At 50 °C the transporter loses about 30% of its cytochalasin B-binding activity/h (A. F. Gibbs & S. A. Baldwin, unpublished work). Stabilization of the transporter structure by cytochalasin B has been reported in a hydrogen-exchange study [44].

The physiological substrate of the transporter, D-glucose, was found to enhance the rate of cleavage of the transporter by trypsin at 25 °C. This finding is reminiscent of the enhanced reactivity of the transporter towards a variety of alkylating agents seen in the presence of D-glucose, and must reflect the presence of a conformation of the transporter in which the trypsin-sensitive peptide bonds are more accessible to the proteinase.

The regions of the transporter that become resistant to thermolytic cleavage upon occupancy of the outward-facing substrate-binding site by bis-mannose derivatives have been suggested to lie in the hydrophobic sequences at the cytosolic ends of membrane-spanning helices 7 and 8 [43]. However, the sites at which this proteinase cleaves the transporter have not yet been definitively established. Similarly, the effects of D-glucose on the rates of alkylation of the transporter by a range of reagents have led to the suggestion that it is the hydrophobic, membrane-spanning portions of the transporter that experience a conformational change [24]. These regions probably include the exofacial cysteine residue(s) that are protected from reaction with maleimides by cytochalasin. The protected residues are known to lie in the *C*-terminal half of the transporter, and probably correspond to Cys-421 and/or Cys-429 in the hydrophobic sequences at the extracellular ends of membrane-spanning helices 11 and 12 [1,40].

In the present study, ethylidene glucose and phloretin were found to reduce the rate of production of both the M_r 45000- and M_r 18000-tryptic fragments of the transporter. This finding implies that both the central hydrophilic region of the sequence and the *C*-terminal region become more resistant to tryptic cleavage upon binding of the inhibitors to the extracellular surface of the transporter. One or both must, therefore, undergo a conformational change, although it is possible that a change at one site sterically hinders the access of trypsin to the other. These hydrophilic regions of the transporter have not previously been identified as undergoing translocation-related conformational changes. Our present findings imply that they have an important role in the mechanism of transport.

We are grateful to the SERC for financial support for this research in the form of a project grant to D.C. and S.A.B.

REFERENCES

1. Mueckler, M., Caruso, C., Baldwin, S. A., Panico, M., Blench, I., Morris, H. R., Lienhard, G. E., Allard, W. J. & Lodish, H. F. (1985) Science **229**, 941–945
2. Chin, J. J., Jung, E. K. Y., Chen, V. & Jung, C. Y. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 4113–4116
3. Pawagi, A. B. & Deber, C. M. (1987) Biochem. Biophys. Res. Commun. **145**, 1087–1091
4. Lee, D. C., Elliot, D. A., Baldwin, S. A. & Chapman, D. (1985) Biochem. Soc. Trans. **13**, 684–685
5. Chin, J. J., Jung, E. K. J. & Jung, C. Y. (1986) J. Biol. Chem. **261**, 7101–7104
6. Alvarez, J., Lee, D. C., Baldwin, S. A. & Chapman, D. (1987) J. Biol. Chem. **262**, 3502–3509
7. Jung, E. K. Y., Chin, J. J. & Jung, C. Y. (1986) J. Biol. Chem. **261**, 9155–9160
8. Deziel, M. R. & Rothstein, A. (1984) Biochim. Biophys. Acta **776**, 10–20
9. Lienhard, G. E., Crabb, J. H. & Ransome, K. J. (1984) Biochim. Biophys. Acta **769**, 404–410
10. Cairns, M., Alvarez, J., Panico, M., Gibbs, A. F., Morris, H. R., Chapman, D. & Baldwin, S. A. (1987) Biochim. Biophys. Acta **905**, 295–310
11. Davies, A., Meeran, K., Cairns, M. T. & Baldwin, S. A. (1987) J. Biol. Chem. **262**, 9347–9352
12. Vidaver, G. A. (1966) J. Theor. Biol. **10**, 301–306
13. Barnett, J. E. G., Holman, G. D., Chalkley, R. A. & Munday, K. A. (1975) Biochem. J. **145**, 417–429
14. Baldwin, S. A. & Lienhard, G. E. (1981) Trends Biochem. Sci. **6**, 208–211
15. Carruthers, A. (1986) J. Biol. Chem. **261**, 11028–11037
16. Carruthers, A. (1986) Biochemistry **25**, 3592–3602
17. Helgerson, A. L. & Carruthers, A. (1987) J. Biol. Chem. **262**, 5464–5475
18. Bowyer, F. & Widdas, W. F. (1958) J. Physiol. (London) **141**, 219–232
19. Krupka, R. M. (1971) Biochemistry **10**, 1143–1148
20. Dawson, A. C. & Widdas, W. F. (1963) J. Physiol. (London) **168**, 644–659
21. Bloch, R. (1974) J. Biol. Chem. **249**, 1814–1822
22. Gorga, F. R. & Lienhard, G. E. (1982) Biochemistry **21**, 1905–1908
23. Appleman, J. R. & Lienhard, G. E. (1985) J. Biol. Chem. **260**, 4574–4578
24. Rampal, A. L. & Jung, C. Y. (1987) Biochim. Biophys. Acta **896**, 287–294
25. Cairns, M. T., Elliott, D. A., Scudder, P. R. & Baldwin, S. A. (1984) Biochem. J. **221**, 179–188
26. Deves, R. & Krupka, R. M. (1978) Biochim. Biophys. Acta **510**, 339–348
27. Baker, G. F. & Widdas, W. F. (1973) J. Physiol. (London) **231**, 143–165
28. Krupka, R. M. (1985) J. Membr. Biol. **83**, 71–80
28a. Gibbs, A. F., Chapman, D. & Baldwin, S. A. (1987) Biochem. Soc. Trans. **15**, 1128–1129
29. Baldwin, S. A., Baldwin, J. M. & Lienhard, G. E. (1982) Biochemistry **21**, 3836–3842

30. Zoccoli, M. A., Baldwin, S. A. & Lienhard, G. E. (1978) J. Biol. Chem. **252**, 6923–6930
31. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
32. Lowe, A. G. & Walmsley, A. R. (1986) Biochim. Biophys. Acta **857**, 146–154
33. Gorga, F. R. & Lienhard, G. E. (1981) Biochemistry **20**, 5108–5113
34. Wheeler, T. J. (1986) Biochim. Biophys. Acta **862**, 387–398
35. Wheeler, T. J. & Whelan, J. D. (1988) Biochemistry **27**, 1441–1450
36. Baldwin, J. M., Gorga, J. C. & Lienhard, G. E. (1981) J. Biol. Chem. **256**, 3685–3689
37. Wheeler, T. J. & Hinkle, P. C. (1981) J. Biol. Chem. **256**, 8907–8914
38. Batt, E. R., Abbott, R. E. & Schachter, D. (1976) J. Biol. Chem. **251**, 7184–7190
39. Roberts, S. J., Tanner, M. A. & Denton, R. M. (1982) Biochem. J. **205**, 139–145
40. Deziel, M. R., Jung, C. Y., Rothstein, A. (1985) Biochim. Biophys. Acta **819**, 83–92
41. Karim, A. R., Rees, W. D. & Holman, G. D. (1987) Biochim. Biophys. Acta **902**, 402–405
42. Kurokawa, T., Tillotson, L. G., Chen, C-C. & Isselbacher, K. J. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 479–482
43. Holman, G. D. & Rees, W. D. (1987) Biochim. Biophys. Acta **891**, 395–405
44. Jung, E. K. Y., Chin, J. J. & Jung, C. Y. (1986) J. Biol. Chem. **261**, 9155–9160

Received 4 May 1988/6 July 1988; accepted 21 July 1988