Biochimica et Biophysica Acta 905 (1987) 295–310
Elsevier

BBA 73803

Investigation of the structure and function of the human erythrocyte glucose transporter by proteolytic dissection

Michael T. Cairns [a], Javier Alvarez [a], Maria Panico [c], Angela F. Gibbs [a], Howard R. Morris [c], Dennis Chapman [a] and Stephen A. Baldwin [a,b]

Departments of [a] Biochemistry and Chemistry, and of [b] Chemical Pathology and Human Metabolism, Royal Free Hospital School of Medicine, (University of London), Rowland Hill Street, London NW3 2PF, and [c] Department of Biochemistry, Imperial College (University of London), London SW7 2AZ (U.K.)

(Received 13 July 1987)

Key words: Glucose transport; Membrane protein; Cytochalasin B; FABMS; Fourier transform infrared spectroscopy; (Human erythrocyte)

Tryptic and papain digestion have been employed to investigate the structure and function of the human erythrocyte glucose transporter. Trypsin cleaves the native protein into two large, membrane-embedded fragments and a number of small peptides that are released from the membrane. These fragments have been isolated and located within the transporter sequence by fast atom bombardment mass spectrometry and amino acid analysis. The results indicate that the segments of the sequence comprising residues 213–269 and 457–492 are cleaved from the cytoplasmic surface of the membrane by trypsin treatment. These findings are compatible with a model previously proposed for the arrangement of the polypeptide in the membrane (Mueckler, M., et al. (1985) Science 229, 941–945). Despite the loss of these 93 residues, the portion of the protein remaining embedded in the membrane is still able to bind cytochalasin B. This binding is inhibited by D-glucose, indicating that the membrane-embedded fragments retain the substrate-binding site. Fourier transform infrared spectroscopic analysis of the protein before and after proteolytic digestion shows that the intramembranous part of the protein is largely α-helical, although some β-sheet structure appears also to be present. The spectroscopic findings also indicate that the extramembranous, cytoplasmic domain of the transporter, which is removed by trypsin, contains α-helical structure.

Introduction

Glucose transport across the human erythrocyte membrane is the best understood of all facilitated diffusion processes. The protein responsible for transport has been purified to near homogeneity, and extensively characterised [2,3]. The isolated protein retains the ability to transport glucose, with kinetics similar to those seen in the intact erythrocyte [4,5]. It also binds the potent inhibitor of transport cytochalasin B [3]. The transporter is a glycoprotein, and migrates as a rather broad band of apparent M_r 55000 on

Abbreviations: SDS, sodium dodecyl sulphate; M_r, relative molecular mass; HPLC, high-pressure liquid chromatography; FAB, fast atom bombardment; FTIR, Fourier transform infrared; dansyl, 5-dimethylamino-1-naphthalene sulphonyl; NTCB, 2-nitro-5-thiocyanobenzoic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; E-64, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane.

Correspondence: S.A. Baldwin, Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, U.K.

SDS-polyacrylamide gels [3,6,7]. Infrared spectroscopic and circular dichroism studies carried out by ourselves and others have shown that the protein is predominantly α-helical in structure, with some random coil conformation, although we have suggested that there may also be some β-structure present [8–10].

Recently, the complete amino acid sequences of the glucose transporters from the human hepatoma cell line HepG2 and from the rat brain were deduced from analyses of cDNA clones [1,11]. The two sequences are almost identical. Structural studies on the human erythrocyte glucose transporter have shown that this protein is probably identical in sequence to the hepatoma protein. Examination of the sequence for hydrophobic segments that could form membrane-spanning α-helices, together with the results of vectorial proteolytic digestion experiments, allowed a model to be proposed for the arrangement of the polypeptide in the membrane [1]. In this model the polypeptide crosses the lipid bilayer 12 times in the form of largely hydrophobic α-helices. The N-terminal region (residues 1–12), the central region (residues 207–271) and the C-terminal region (residues 451–492) of the protein are predicted to lie on the cytoplasmic side of the membrane. We have recently confirmed the cytoplasmic location of the C-terminus through the use of antibodies raised against a synthetic peptide corresponding to residues 477–492 [12].

The aim of the work described in the present paper was to obtain direct evidence for the location of the proposed extramembranous segments of the protein through examination of their susceptibility to proteolytic cleavage. The native transporter is known to be susceptible to tryptic cleavage only at the cytoplasmic surface of the membrane [13,14], cleavage at several places near the mid-point and near the C-terminus of the sequence yielding two large membrane-bound fragments [15,16]. One of the fragments is glycosylated, and migrates as a broad band of apparent M_r 23000–42000 on SDS-polyacrylamide gels. We have recently shown, using antibodies raised against a synthetic peptide, that this fragment is derived from the N-terminal half of the protein [12]. The other fragment migrates as a sharp band of apparent M_r 18000, and is derived from the C-terminal half of the protein [12,15,16]. If the protein is photoaffinity labelled with cytochalasin B before digestion, the label is found solely associated with this latter fragment, suggesting that it encompasses a part of the inhibitor binding site [15,16].

In this paper we identify the sites of tryptic cleavage that give rise to the two large tryptic fragments of the transporter. Tryptic cleavage of the protein has also enabled us to examine the separate roles and structures of the intramembranous and extramembranous portions of the transporter sequence.

A preliminary report of some of these findings was presented in the form of a poster at the 616th meeting of the Biochemical Society, 1985 (Biochem. Soc. Trans. (1986) 14, 747–748).

Materials and Methods

Materials

Diphenylcarbamyl chloride-treated trypsin, papain, ^2H_2O and iodoacetamide were obtained from Sigma (Poole, Dorset, U.K.). Bovine lung aprotinin and the papain inhibitor E-64 were obtained from the Boehringer Corporation (London) Ltd., (Lewes, East Sussex, U.K.). Pre-stained molecular weight standards for SDS-polyacrylamide gel electrophoresis were purchased from Bio-Rad (Watford, U.K.). [4-^{3}H]Cytochalasin B (10.3 Ci/mmol) was obtained from New England Nuclear (Southampton, U.K.). Trifluoroacetic acid was supplied by Rathburn Chemicals Ltd. (Walkerburn, Peebleshire, U.K.) and HPLC grade acetonitrile by Koch-Light Ltd., (Genzyme) (Haverhill, Suffolk, U.K.). Ammonium acetate for HPLC was provided by the Aldrich Chemical Co. Ltd. (Gillingam, Dorset, U.K.). Outdated human blood was provided by the blood bank of the Royal Free Hospital.

Preparation and assay of alkali-stripped erythrocyte membranes and the purified glucose transporter

Alkali-stripped human erythrocyte membranes were prepared by the method of Gorga and Lienhard [17]. The human erythrocyte glucose transporter was purified and reconstituted in erythrocyte membrane lipids by the method of Baldwin et al. [3] with the modifications introduced by Cairns

et al. [15]. Its cytochalasin B binding activity before and after proteolytic digestion was measured by equilibrium dialysis using $4 \cdot 10^{-8}$ M [4-^{3}H]cytochalasin B as previously described [18]. Under these conditions the ratio of bound to free cytochalasin B is approximately equal to the concentration of binding sites divided by the dissociation constant for cytochalasin B, and is referred to as the cytochalasin B binding activity. For accurate determination of the dissociation constant and the concentration of binding sites, the binding was measured over a range of cytochalasin B concentrations and the data analysed by the LIGAND procedure of Munson and Rodbard [19]. Protein was measured by the procedure of Lowry et al. [20] except that 0.5% (w/v) SDS was included to solubilise membranous samples. Proteolytic digests of the transporter were analysed by SDS-polyacrylamide gel electrophoresis, using either the procedure of Laemmli [21] and 12% acrylamide gels, or the gradient gel method of Hashimoto et al. [22].

Tryptic digestion of alkali-stripped erythrocyte membranes

In order to examine the effect of tryptic cleavage of the tranpsorter on its cytochalasin B binding activity, alkali-stripped erythrocyte membranes at a protein concentration of 2 mg/ml in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA (pH 7.4) were digested with 50 μg/ml trypsin at 25°C. After 16 h, digestion was halted by the addition of 100 μg/ml bovine lung aprotinin.

Isolation of the large tryptic fragments of the transporter

Purified glucose transporter (275 μg/ml) in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA (pH 7.4) was incubated at 25°C with 4 μg/ml trypsin. Two further identical additions of trypsin were made after two and four hours. After six hours digestion was halted by the addition of 24 μg/ml bovine lung aprotinin. The digest was then dialysed against 100 mM Tris-HCl, 1 mM EDTA (pH 8.3) and concentrated to 2.75 mg/ml by centrifugation for 1 h at 126000 × *g* (r_{av} 5.6 cm) at 4°C. Dithiothreitol (1 mM) was added and the mixture incubated for 30 min at 25°C under nitrogen. SDS was next added to give a concentration of 1.1% (w/v) and the mixture incubated for a further 30 min under nitrogen. Thiol groups were then alkylated by the addition of 10 mM iodoacetamide followed by incubation for a further hour in the dark under nitrogen at 25°C. Excess reagents were then removed by the addition of 2-mercaptoethanol and dialysis at 4°C against 50 mM Tris-HCl (pH 7.4).

The large tryptic fragments were then separated by electrophoresis on a 3 mm thick, 12% acrylamide gel in SDS, according to the procedure of Laemmli [21]. The fragments were located by reference to pre-stained molecular weight standards, and excised from the gel. They were then recovered from the gel pieces by electroelution using a Bio-Trap apparatus from Schleicher and Schuell, according to the manufacturers instructions. After extensive dialysis against distilled water, samples were taken for analytical gel electrophoresis and for acid hydrolysis.

Isolation of the small tryptic fragments of the transporter

Purified glucose transporter (2 mg/ml) in 0.5% (w/v) ammonium bicarbonate (pH 8.0) was incubated at 25°C with 20 μg/ml trypsin. A second identical addition of trypsin was made after 2 h. After 4 h the digestion was halted by lyophilisation. The lyophilised digest was extracted three times with chloroform/water (1 : 1, v/v) to remove lipids, and then lyophilised again. Peptides soluble in 0.1% (v/v) trifluoroacetic acid were separated by reverse phase HPLC on a C-18 column (300 Å pore size; Aquapore RP-300, Brownlee) using a gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid. An Altex HPLC system with model 420 solvent programmer was used. Peaks, detected by absorption at 220 nm, were collected and vacuum-dried. Some peptides were redissolved in a small volume of 25 mM ammonium acetate (pH 6.0) and then rechromatographed on the same column using a gradient of acetonitrile in 25 mM ammonium acetate (pH 6.0).

In some experiments, the transporter preparation (275 μg/ml) was centrifuged at 126000 × *g* (r_{av} 5.6 cm) for 1 h at 4°C after tryptic digestion (2.75 μg trypsin per ml added initially and again after 2 h during a 4 h digestion), to separate

soluble from membrane-associated tryptic fragments. The supernatant was lyophilised then dissolved in 0.1% (v/v) trifluoroacetic acid directly for HPLC. The membranous pellet was delipidated as described above before analysis by HPLC.

Peptide characterisation

Fast atom bombardment (FAB) mass spectrometry of both peptide mixtures and individual peptides was caried out using a V.G. ZAB high-field instrument equipped with an M-SCAN fast atom bombardment ion gun as previously described [23,24]. Mass spectra were recorded up to a mass of 3000 a.m.u. at full accelerating voltage. In order to confirm the assignment of signals in the spectra to particular peptides, soluble peptides in the tryptic digest of the transporter were subjected to up to two rounds of Edman degradation before analysis. The digest (approximately 5 nmol of each peptide) in 100 μl H_2O and 200 μl pyridine was incubated at 45°C for 1 h with 10 μl phenylisothiocyanate. After extraction with toluene and drying, the phenylthiocarbamyl peptides were cleaved with trifluoroacetic acid at 52°C for 15 min, dried in vacuo, then extracted with butyl acetate before analysis by mass spectrometry.

For amino acid analysis, peptides were hydrolysed for 24–96 h at 110°C in 6 M HCl, containing 0.04% (v/v) 2-mercaptoethanol and 0.04% (v/v) phenol. Analysis of the hydrolysates was performed on an LKB 4151 Alpha Plus analyser linked to a Trilab 2000 multi-channel chromatography data system. N-terminal analysis of the peptides was carried out by dansylation [25].

Infrared spectroscopy

For infrared spectroscopic studies, the purified glucose transporter (180 μg/ml) in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA (pH 7.4) was incubated for 2 h at 25°C with 20 μg/ml trypsin. A second, identical addition of trypsin was then made, and the incubation continued for another 16 h. Digestion was halted by centrifugation and resuspension of the membranes twice in 5 mM Hepes, 160 mM NaCl (pH 7.4). For papain treatment, reconstituted glucose transporter at 1.8 mg/ml in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, 10 mM L-cysteine (pH 7.4) was incubated with 0.5 mg/ml papain at 37°C. The digestion was terminated after 2 h or 3 days by the addition of 0.1 mM E-64, and the membranes washed and resuspended in 5 mM Hepes, 160 mM NaCl (pH 7.4).

For infrared spectroscopy, samples of intact or protease-digested transporter were centrifuged then resuspended in 5 mM Hepes, 160 mM NaCl, (pH 7.4) at a protein concentration of 10–15 mg/ml. For deuterium exchange, the reconstituted transporter was lyophilised at −60°C, 7 mmHg from a suspension in water and then resuspended in 5 mM Hepes, 160 mM NaCl (p^2H 7.4) in ^2H_2O. The cytochalasin B binding activity of the transporter treated in this way was > 90% of that for an untreated sample. Buffer and protein samples were scanned under the same temperature and scanning conditions in a Perkin-Elmer FTIR 1750 spectrometer, using a Perkin-Elmer 7300 data station for data acquisition and analysis. Samples were placed in a thermostatted Beckman FH-01 CFT microcell fitted with CaF_2 windows and a 6 μm tin spacer. Each sample was equilibrated at 20°C for 15 min, and 200 scans with a resolution of 2 cm^{-1} were then averaged for a period of 1 h. A sample shuttle was used to average the background spectrum over the period of data collection. The spectrometer was continuously purged with dry air to eliminate interference from water vapour absorptions in the amide regions of the spectra. Difference spectra were obtained by digitally subtracting buffer spectra from the corresponding sample spectra. Second derivative spectra were generated from the difference spectra by using an OBEY program available from Perkin-Elmer. Noise in the second derivative spectra was reduced by smoothing over a 13 data point range (13 cm^{-1}). Spectra were deconvoluted using the Perkin-Elmer ENHANCE function, which is analogous to the method developed by Kauppinen et al. [26]. Deconvolution parameters σ (half-width at half-height) and K (relative reduction in band width) are given in the figure legends. Because the protein concentration in the various samples analyzed differed, the absorbance scales used in Figs. 4, 5 and 7 for each sample differ, and are indicated in the legends.

Results

For isolation of small tryptic peptides from the glucose transporter, digestion was performed in the volatile buffer, ammonium bicarbonate. The cytochalasin B binding activity of the transporter in this buffer was comparable to that in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA (pH 7.4). Upon digestion with trypsin, the cytochalasin B binding activity was decreased to the same extent as previously reported for digestion in the phosphate buffer [15] (data not shown). In addition, the digest yielded a pattern of fragments on an SDS-polyacrylamide gel identical to that previously reported for digestion in the non-volatile buffer [15]: two fragments were seen, one a sharp band of apparent M_r 18000 and the other a broad band of apparent M_r 23000–42000 (data not shown).

Reverse-phase HPLC of the peptides from the delipidated digest which were soluble in 0.1% (v/v) trifluoroacetic acid yielded the separation shown in Fig. 1. Peptides A, B, E, F, G, H and I yielded a single N-terminal amino acid by dansylation (Table I). From the masses of the quasi-molecular ions $[M + H]^+$ determined by FAB mass spectrometry and from their amino acid compositions it was possible to locate each peptide in the amino acid sequence of the transporter (Table I). Amino acid analysis indicated that peaks C, D and J were not pure, and so they were rechromatographed using the ammonium acetate/acetonitrile system described in Materials and Methods. The major components of peaks D and J were purified by this procedure, and could be located in the transporter sequence by mass spectrometry, N-terminal analysis and amino acid analysis (Table I). Peptide C was obtained in low yield, so that insufficient material was available for mass spectrometry and N-terminal analysis. However, the amino acid composition of the peptide suggested that it comprised residues 265–269 of the transporter (Table I).

In order to determine whether the small tryptic peptides were actually released from the membrane during digestion, or remained attached to the remainder of the transporter, another tryptic digest was fractionated by centrifugation at 126000 × g (r_{av} 5.6 cm) for 1 h at 4°C. Analysis of the supernatant by HPLC yielded a pattern of peptides identical to that shown in Fig. 1 (data not shown). The pellet, containing the membrane-

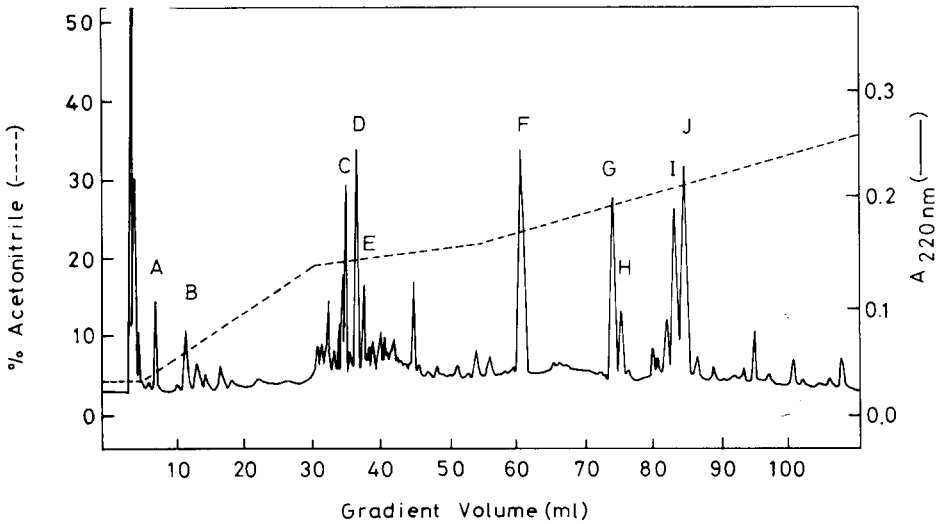

Fig. 1. Separation of the small tryptic peptides of the transporter by reverse-phase HPLC. Purified glucose transporter (2 mg/ml) in 0.5% (w/v) ammonium bicarbonate was incubated at 25°C with 20 μg/ml trypsin. A second addition of trypsin was made after 2 h, and incubation continued for a further 2 h. After extraction of lipid, the peptides in the digest soluble in 0.1% (v/v) trifluoroacetic acid were separated using the illustrated gradient (— — —) of acetonitrile in 0.1% (v/v) trifluoroacetic acid, as described in Materials and Methods. Peptides were detected by their absorbance at 220 nm (———).

TABLE I

PROPERTIES OF PEPTIDES ISOLATED FROM A TRYPTIC DIGEST OF THE HUMAN ERYTHROCYTE GLUCOSE TRANSPORT PROTEIN

Amino acid	mol residues/mol peptide [a] in peptide									
	H	B	E	F	A	D	C	G	I	J
Asx	1.4 (1)	2.2 (2)	–	1.8 (2)	–	–	–	0.9 (1)	1.9 (2)	1.2 (1)
Thr	–	–	–	1.6 (2)	–	–	–	0.6 (1)	1.0 (1)	0.9 (1)
Ser	–	–	0.6 (1)	–	0.8 (1)	–	0.9 (1)	0.6 (1)	2.1 (3)	1.0 (1)
Glx	–	2.3 (2)	–	2.0 (2)	2.0 (2)	1.2 (1)	–	1.0 (1)	5.2 (5)	3.0 (3)
Pro	–	–	–	–	–	–	1.1 (1)	–	2.1 (2)	1.7 (2)
Gly	–	–	–	1.4 (1)	–	–	–	0.9 (1)	2.9 (3)	1.3 (1)
Ala	–	–	–	1.1 (1)	–	–	1.3 (1)	0.9 (1)	1.9 (2)	1.0 (1)
Val	–	–	0.9 (1)	1.1 (1)	–	–	–	–	1.1 (1)	1.3 (1)
Met	–	–	–	0.7 (1)	–	1.6 (2)	–	–	–	–
Ile	1.1 (1)	–	–	–	–	–	–	0.6 (1)	–	–
Leu	2.1 (2)	–	0.9 (1)	1.2 (1)	–	–	–	–	2.4 (2)	1.8 (2)
Tyr	–	–	–	–	–	–	1.0 (1)	–	–	–
Phe	1.0 (1)	–	–	–	–	–	–	0.9 (2)	1.2 (1)	1.0 (1)
Lys	–	–	1.0 (1)	1.0 (1)	–	–	–	–	1.0 (1)	0.3
His	–	–	–	0.9 (1)	–	–	–	–	1.0 (1)	0.8 (1)
Arg	1.0 (1)	1.0 (1)	–	–	1.0 (1)	1.0 (1)	1.0 (1)	0.4 (1)	–	–
N-terminus [b]	Phe	Asx	Ser	Gly	Glx	Glx	n.d.	Thr	Glx	n.d.
$[M+H]^+$ [c]	775	661	446	1444	520	565	n.d.	1142	2497	1639
Location in sequence [d]	213–218	219–223	226–229	233–245	246–249	250–253	265–269	459–468	469–492	478–492

[a] Peptides were hydrolysed for 24 h before analysis. Values in parenthesis are those expected for each peptide.
[b] Determined by dansylation. n.d., not determined.
[c] From FAB mass spectrometry.
[d] Predicted location in the erythrocyte protein, based on the hepatoma protein sequence [1].

bound fragments, yielded no peptides soluble in 0.1% (v/v) trifluoroacetic acid after delipidation. Therefore, all the peptides described above must have been released from the membrane during digestion. In order to ensure that all the small tryptic peptides liberated from the transporter had been identified, the tryptic digest supernatant was also examined by FAB mass spectrometry, yielding the quasi-molecular ions listed in Table II. In most cases the assignments of the signals to particular regions of the transporter sequence were confirmed by the changes in mass observed after one or two rounds of Edman degradation carried out on the peptide mixture (Table II). Although many of the signals corresponded to peptides that had been isolated by HPLC, others were derived from overlapping peptides and one (m/z 990) represented a region of the sequence (residues 257–264) that had not previously been detected in the digest.

Preparative gel electrophoresis was used successfully in the isolation of the large, membrane-bound tryptic fragments of the glucose transporter (Fig. 2). Samples of each fragment were hydrolysed in duplicate for 24, 48 or 96 h and then subjected to amino acid analysis. The amino acid compositions of the fragments are given in Table III. That of the glycosylated fragment was found to correspond reasonably closely to that predicted for residues 1–212, although the yields of some amino acids such as methionine, serine and isoleucine were lower than expected. These findings may have resulted from oxidative damage to the polypeptide during electrophoresis and from incomplete hydrolysis of some of the peptide bonds even after 96 h. The N-terminus of the fragment could not be identified by dansylation, possibly because of its modification during isolation of the polypeptide. However, the ratio of arginine to lysine determined by amino acid analysis, 1.24,

TABLE II

IDENTIFICATION OF PEPTIDES IN A TOTAL TRYPTIC DIGEST OF THE TRANSPORTER BY FAB-MASS SPECTROMETRY

m/z	Assignment in the amino acid sequence [a]	
416	230–232	: N-terminal Lys or Gln after 1 cycle of Edman
446	8–11 or 226–229	
574	7–11 or 226–230	
775	213–218	: N-terminal Phe then Leu or Ile confirmed after two cycles of Edman
990	257–264	: N-terminal Val then Thr confirmed after two cycles of Edman
1142	459–468	: N-terminal Thr then Phe confirmed after two cycles of Edman
1444	233–245	: N-terminal Gly then Thr confirmed after two cycles of Edman
1639	478–492	
1713	231–245	: N-terminal Leu or Ile then Arg confirmed after two cycles of Edman
1945	233–249	: N-terminal Gly confirmed after one cycle of Edman
2480	469–492	: cyclized Gln
2497	469–492	: N-terminal Gln confirmed after one cycle of Edman

[a] Assignments were confirmed in many cases by the change in mass seen following one or two cycles of Edman degradation. Residue numbers refer to the sequence of the human hepatoma glucose transporter [1].

was close to that of 1.33 predicted for a fragment containing residues 1–212. If the fragment had arisen by cleavage at residues 6, 7 or 11, arginine to lysine ratios of 1.60, 2.00 and 1.75, respectively, should have been obtained.

The amino acid composition of the M_r 18000 fragment corresponded reasonably well to that predicted for residues 270–456, although some amino acids were obtained in low yield, as was found for the glycosylated fragment. The N-terminus of the fragment could not be identified also, possibly because of cyclization of Gln-270. However, the ratio of arginine to lysine determined by amino acid analysis, 1.33, was identical to that predicted for a fragment containing residues 270–456. If the fragment had arisen by cleavage at residues 264, 451 or 458, ratios ranging from 1.67 to 2.50 would have been expected.

In a previous study we showed that tryptic cleavage of the glucose transporter lowered the affinity, but did not abolish the binding of cyto-

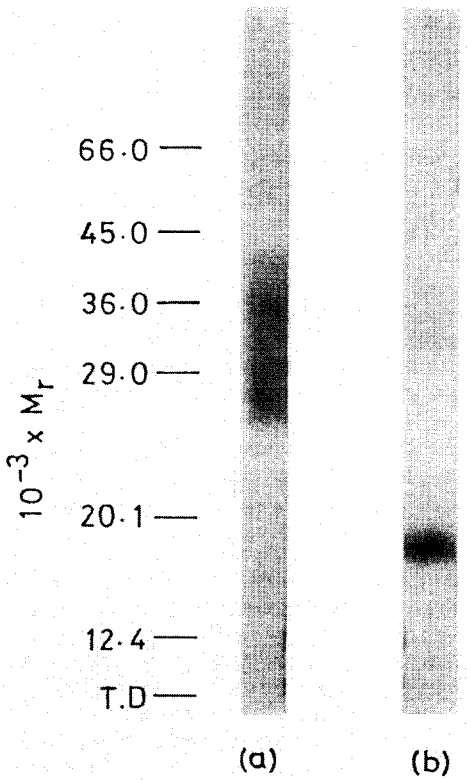

Fig. 2. SDS-polyacrylamide gel electrophoresis of the large tryptic fragments of the transporter. Samples of (a) the glycosylated and (b) the non-glycosylated large tryptic fragments of the glucose transporter, isolated by preparative gel electrophoresis, were electrophoresed on a 12% SDS-polyacrylamide gel and then stained with Coomassie blue. The positions of the following molecular weight markers are indicated: bovine serum albumin (M_r 66000); ovalbumin (M_r 45000); glyceraldehyde-3-phosphate dehydrogenase (M_r 36000); carbonic anhydrase (M_r 29000); soyabean trypsin inhibitor (M_r 20100) and cytochrome c (M_r 12400). TD, tracking dye.

TABLE III

AMINO ACID COMPOSITION OF THE LARGE TRYPTIC FRAGMENTS OF THE GLUCOSE TRANSPORTER

Amino acid	Residues/fragment [a]			
	M_r 23000–42000 fragment [e]		M_r 18000 fragment [e]	
Cys [b]	2.1 ± 0.2	(3)	2.3 ± 0.1	(3)
Asx	10.6 ± 0.2	(9)	5.6 ± 0.1	(4)
Thr	10.0 ± 0.3 [c]	(11)	8.8 ± 0.3 [c]	(10)
Ser	11.7 ± 0.5 [c]	(17)	12.3 ± 0.4 [c]	(11)
Glx	13.8 ± 0.4	(13)	14.1 ± 0.2	(17)
Pro	8.1 ± 0.3	(10)	8.6 ± 0.3	(10)
Gly	20.5 ± 0.2	(25)	14.2 ± 0.3	(15)
Ala	11.9 ± 0.2	(10)	14.9 ± 0.4	(15)
Val	15.8 [d]	(19)	16.8 [d]	(21)
Met	5.2 ± 0.2	(10)	3.0 ± 0.1	(4)
Ile	12.1 [d]	(16)	12.8 [d]	(18)
Leu	26.6 [d]	(30)	19.2 [d]	(19)
Tyr	4.9 ± 0.02	(5)	5.5 ± 0.1	(7)
Phe	11.5 ± 0.2	(15)	15.0 [d]	(19)
His	2.8 ± 0.1	(2)	1.5 ± 0.04	(1)
Lys	7.9 ± 0.2	(6)	5.7 ± 0.1	(3)
Arg	9.8 ± 0.5	(8)	7.6 ± 0.3	(4)

[a] Unless noted, mean ± S.D. for six determinations (duplicate 24, 48 and 96 h hydrolyses). Compositions calculated on the assumption that Σ Glx, Gly, Ala, Arg = 56 residues for the fragment of apparent M_r 23000–42000, and that Σ Glx, Gly, Ala, His, Lys, Arg = 58 residues for the fragment of apparent M_r 18000.

[b] Determined as *S*-(carboxymethyl)cysteine.

[c] Extrapolated to 0 h hydrolysis, ± S.E.

[d] Means of duplicate values from hydrolysis for 96 h.

[e] Values in parenthesis are those expected for fragments comprising residues 1–212 and 270–456, respectively.

chalasin B to the residual, membrane-bound fragments of the protein [15]. However, we did not investigate whether this binding was still sensitive to inhibition by D-glucose. Investigation of this question in the present study made use of alkali-stripped erythrocyte membranes, in which the glucose transporter comprises about 10% of the total protein and which are known to be unsealed [17]. Tryptic digestion of these membranes as described in Materials and Methods was found to cleave essentially all of the glucose transporter molecules present, as revealed by Western blotting using anti-transporter antibodies (data not shown). Scatchard plot analysis of the binding of cytochalasin B to the trypsin-treated membranes showed that they still bound cytochalasin B (Fig. 3). The data gave a good fit to a model comprising a single set of high-affinity binding sites together with a non-specific component. Trypsin treatment decreased the association constant for binding from 4.96 ± 0.72 (S.E.) μM^{-1} to 3.32 ± 0.29 (S.E.) μM^{-1} without affecting the number of cytochalasin B binding sites, a result similar to that previously reported for tryptic digestion of the purified transporter [15]. In the presence of 30 mM D-glucose the apparent association contant was reduced further, to 0.19 ± 0.10 (S.E.) μM^{-1}, indicating that glucose inhibited the binding with an apparent K_i of about 2 mM (Fig. 3). This value was somewhat smaller than the apparent K_i of 20 mM measured for inhibition by D-glucose of binding to undigested membranes (data not shown), indicating that glucose binds with higher affinity to the trypsin-treated than to the undigested transporter.

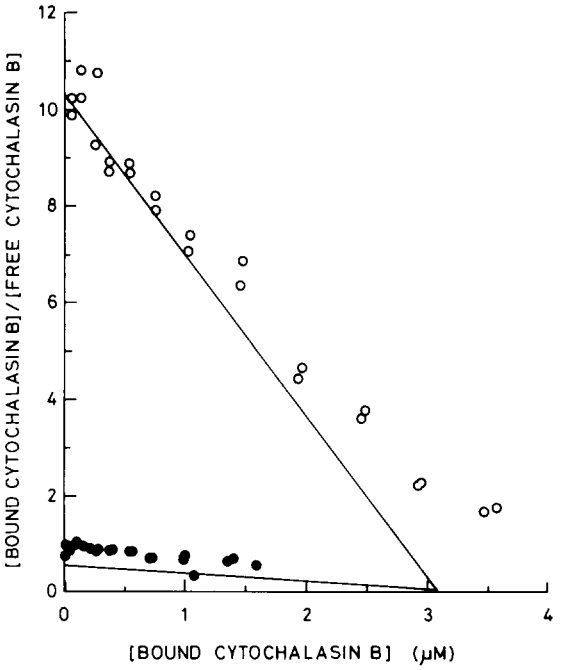

Fig. 3. Scatchard plot analysis of cytochalasin B binding to trypsin-digested, alkali-stripped erythrocyte membranes. Binding to trypsin-digested erythrocyte membranes (1.8 mg protein/ml) was measured in the absence (○) and presence (●) of 30 mM D-glucose. The data points shown are not corrected for non-specific binding. Straight lines are computerised best fits for the specific component of binding, after correction for non-specific binding by the LIGAND procedure [19].

The ability of the trypsin-treated transporter to bind cytochalasin B in a D-glucose-inhibitable

manner suggested that the structure of the membrane-bound fragments had not been greatly altered by tryptic cleavage. It was therefore felt that information about the structure, in the intact transporter, of both these regions of the sequence and those released by trypsin, could be gained by comparing the FTIR spectra of the intact and the trypsin-treated protein. Fig. 4 shows the effect of tryptic digestion on the infrared spectrum of the glucose transporter in H_2O solution. Because the transporter was repeatedly washed by centrifugation before spectroscopy, only the membrane-bound fragments of the protein were present. Second-derivative spectra show that the same bands are present before and after treatment, indicating that the same types of structure are present in both cases. Deconvolution, on the other hand, shows that the relative intensity of the bands is different in both cases. Absorption at about 1630 cm^{-1}, corresponding to β-sheet structure, is stronger in the trypsin-treated sample than in the intact transporter when compared with the absorption at 1657 cm^{-1}, which corresponds to both α-helical and random coil structure. This observation suggests that those portions of the transporter that are released as soluble peptides upon tryptic digestion include mainly either α-helical or random coil segments.

Random coil and α-helical structure can usually be distinguished by hydrogen-deuterium exchange: the frequency at which α-helical structure absorbs barely alters upon exchange [10,27], but the random coil structure band shifts to considerably lower wavenumbers (some 15–20 cm^{-1}). In the case of the glucose transporter, a strong band remains after exchange at 1657 cm^{-1}, consistent

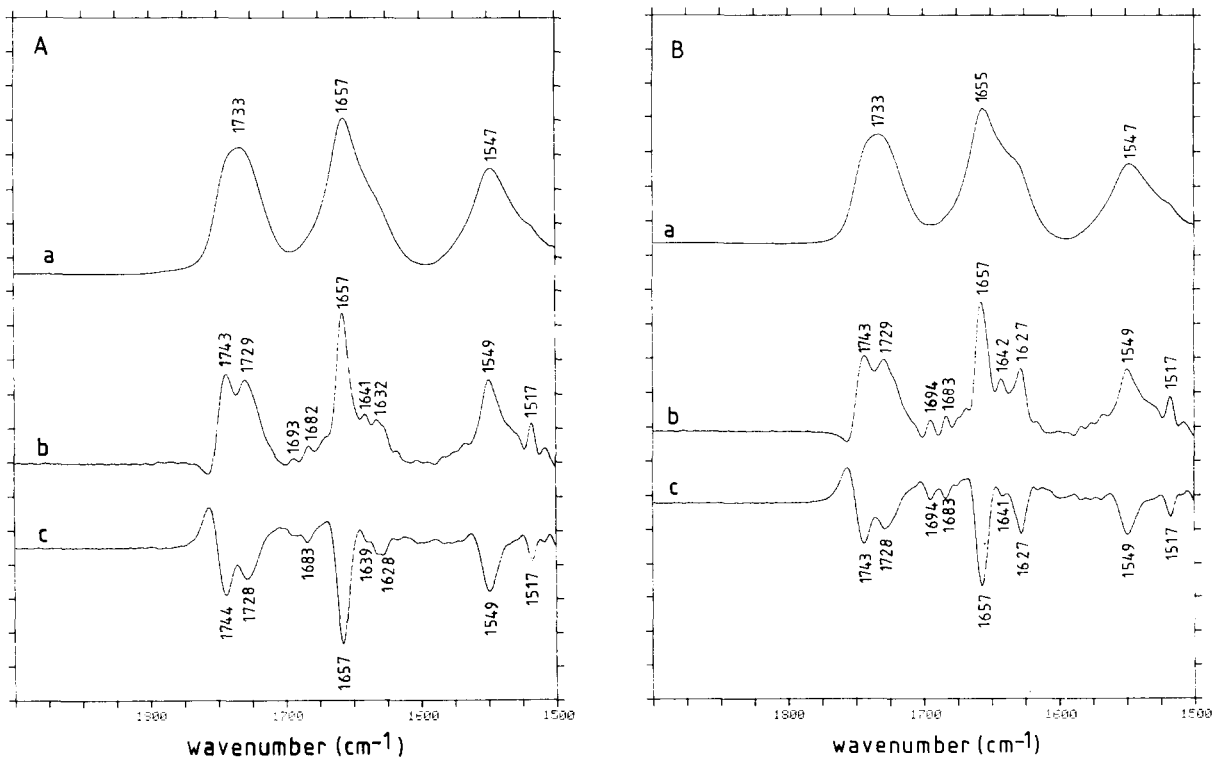

Fig. 4. FTIR spectra of the native and trypsin-digested glucose transporter in H_2O buffer. Spectra of (A) the native and (B) the trypsin-digested glucose transporter were recorded in 5 mM Hepes, 160 mM NaCl (pH 7.4). 200 scans were averaged at 2 cm^{-1} resolution for both the sample and the buffer at 20 °C. (a) Difference spectra; (b) spectra deconvoluted using parameters $\sigma = 10$ cm^{-1}, $K = 2.75$; (c) second derivative spectra. The ordinate scale represents absorbance for (a) and (b), and absorbance/wavenumber^2 for (c). Scale divisions in (A) are 0.030 and 0.060 absorbance units for (a) and (b), respectively, and 0.015 absorbance units/cm^{-2} for (c). Scale divisions in (B) are 0.023 and 0.045 absorbance units for (a) and (b), respectively, and 0.011 absorbance units/cm^{-2} for (c).

with the presence of α-helical secondary structure (Fig. 5A). There also appears to be an increase in intensity near 1641 cm^{-1} (see Fig. 5A (b)), indicating that there may be some random coil structure. After digestion with trypsin, a relative increase in the intensity of the band at around 1630 cm^{-1} with respect to the absorption at both 1656 cm^{-1} and 1644 cm^{-1} can be seen (Fig. 5). This indicates that tryptic digestion has removed mainly α-helical structure and perhaps some random coil structure.

From the studies described above concerning the sites of tryptic cleavage of the transporter, it is clear that the membrane-bound fragments of the transporter may contain a considerable number of extramembranous residues, in loops connecting the membrane-spanning segments of the protein. The apparent presence of β-structure revealed by FTIR spectroscopy of the trypsin-treated transporter might therefore result either from the intramembranous or extramembranous portions of the two large fragments. In an attempt to localise this structure, more vigorous digestion of the transporter was carried out using the less specific protease papain. Digestion of the transporter with this enzyme for 2 h or 3 days, as described in Materials and Methods, decreased the cytochalasin B binding activity by about 75% and by 90%, respectively. These values can be compared to the loss of 75% seen upon tryptic digestion by the procedure described in Materials and Methods.

The pattern of fragments seen upon SDS-polyacrylamide gels of the transporter after 2 h of papain digestion resembled that seen with trypsin. On a 12% acrylamide gel, the digest showed a broad band of apparent M_r 23000–42000, and a sharp band of apparent M_r 20000 (Fig. 6). A number of less intense bands of lower apparent M_r were also present. Upon gradient gel electrophoresis, a broad band of apparent M_r 17000 to

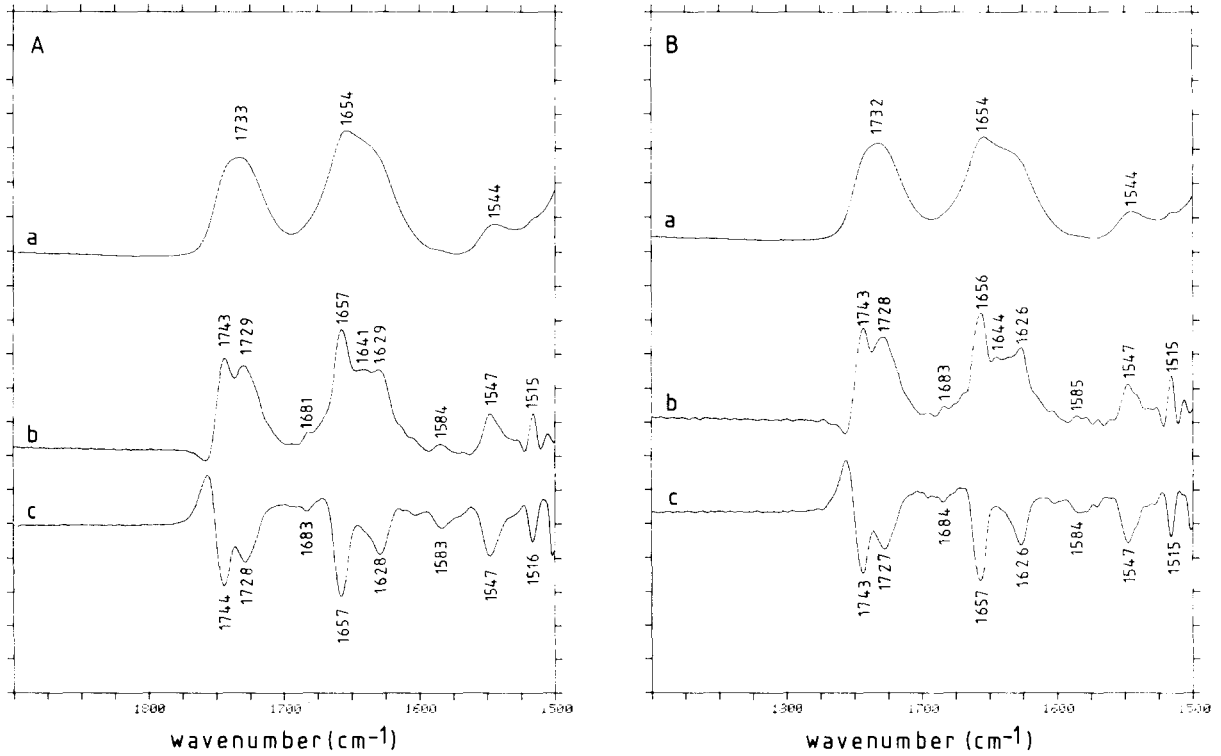

Fig. 5. FTIR spectra of the native and trypsin-digested glucose transporter in ^2H_2O buffer. Spectra of (A) the native and (B) the trypsin-digested glucose transporter were recorded in 5 mM Hepes, 160 mM NaCl (p^2H 7.4). (a) Difference spectra, (b) deconvoluted spectra, (c) second derivative spectra. Scale divisions in (A) are 0.023 and 0.035 absorbance units for (a) and (b), respectively, and 0.006 absorbance units/cm^{-2} for (c). Other details were as described for Fig. 4.

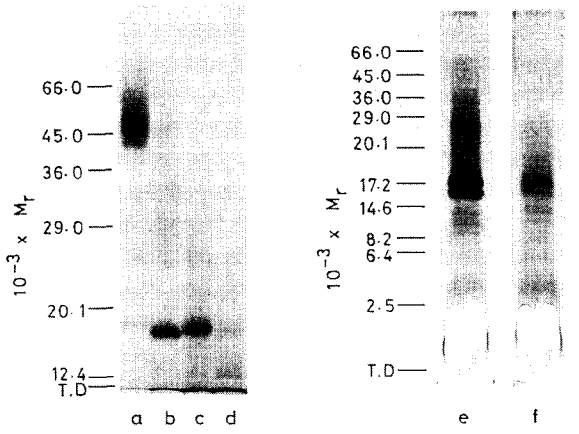

Fig. 6. SDS-polyacrylamide gel electrophoresis of the glucose transporter and the fragments produced by digestion with trypsin and papain. Cleavage with trypsin and papain was carried out as described in Materials and Methods. Samples (a)–(d) were electrophoresed on a 12% acrylamide gel, samples (e) and (f) on a gel containing gradient of 10–18% acrylamide. The gels were stained with Coomassie blue. The positions of marker proteins of known M_r, described in the legend to Fig. 2, are indicated. In addition, myoglobin and its cyanogen bromide fragments were used as markers: these had M_r values of 17200, 14632, 8235, 6383, and 2556, respectively. (a) Intact transporter, (b) trypsin-digested transporter, (c) and (e) transporter digested with papain for 2 h, (d) and (f) transporter digested with papain for 3 days.

29000 was seen, corresponding to the glycosylated tryptic fragment of the transporter (Fig. 6). The major sharp band seen on 12% acrylamide gels was resolved on gradient gels into two bands with apparent M_r values of 16000 and 17000. In addition, less intense sharp bands were present with apparent M_r values of 12000, 10000 and 9000 (Fig. 6). A broad band of apparent M_r 3000–4000 was also present.

Digestion of the transporter with papain for three days led to the almost complete disappearance from the gel of the fragments of M_r 16000 and greater (Fig. 6). There was a corresponding increase in the staining intensity of the lower M_r fragments, particularly those that migrated as a diffuse band of apparent M_r 3000–4000 (Fig. 6). Unfortunately, the presence of lipids in the samples distorted the pattern of these fragments which migrated near the dye front, and so precluded accurate estimation of their relative molecular mass.

The FTIR spectrum of the transporter digested with papain for 2 h resembled that seen after tryptic digestion (Figs. 4, 5 and 7). With the membrane in ^2H_2O, both the α-helical band seen at 1657 cm^{-1} and the random coil absorption at about 1645 cm^{-1} were decreased in intensity relative to the β-structure band at about 1630 cm^{-1} (Fig. 7). However, despite the much more extensive fragmentation of the protein produced by papain digestion for 3 days (Fig. 6), little further change in the FTIR spectrum was seen (Fig. 7). The presence of a strong band at 1657 cm^{-1} in ^2H_2O, together with bands at 1691, 1679, 1641 and 1630 cm^{-1} in the spectrum in H_2O indicated that the residual membrane-bound protein still retained both α-helical and β-sheet structure.

Conclusions

Tryptic digestion of the native, membrane-bound glucose transporter from the human erythrocyte membrane was found to yield a number of small, soluble peptides in addition to two large, membrane-bound fragments. By a combination of HPLC, N-terminal and amino acid analysis, and FAB mass spectrometry, peptides derived from regions 213–223, 226–253, 257–269 and 459–492 were identified in the supernatant of a tryptic digest of the transporter after removal of the membranes by centrifugation. Peptides derived from residues 224–225 (Ala-Lys) and from residues 254–256 (Glu-Lys-Lys) were not identified: these small hydrophilic peptides probably were not retained at all by the HPLC column and so could not be separated. The dipeptide Gly-Arg, corresponding to residues 457 and 458 was likewise not identified. However, its presence in the digest could be inferred from the fact that the large tryptic fragment of apparent M_r 18000 was shown by amino acid analysis, and in particular by its Arg/Lys ratio, to comprise residues 270 to 456 of the transporter sequence. Similarly, the overall amino acid composition and more particularly the Arg/Lys ratio of the large glycosylated tryptic fragment of the transporter of apparent M_r 23000–42000 indicated that it comprised residues 1–212.

Fig. 8 shows the model we previously proposed for the arrangement of the transporter polypeptide

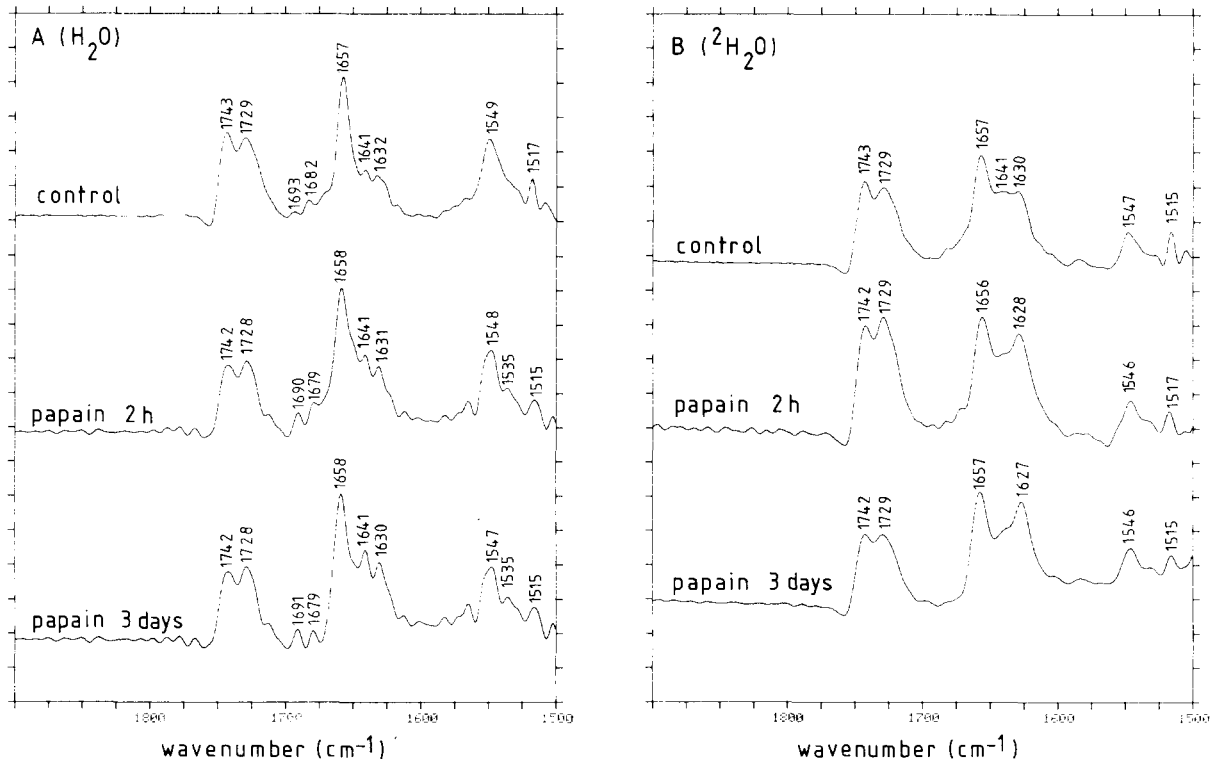

Fig. 7. FTIR spectra of the native and papain-digested glucose transporter in H_2O and ^2H_2O buffers. Spectra were recorded in (A) 5 mM Hepes, 160 mM NaCl (pH 7.4) or (B) 5 mM Hepes, 160 mM NaCl (p^2H 7.4). Control spectra were deconvoluted using parameters $\sigma = 10\ cm^{-1}$, $K = 2.25$. Scale divisions in (A) are 0.065, 0.030 and 0.026 absorbance units for the control, the 2 h papain-digested and the 3 day papain-digested samples, respectively. Scale divisions in (B) are 0.040, 0.002 and 0.006 absorbance units for the control, the 2 h papain-digested and the 3 day papain-digested samples, respectively.

in the membrane, with potential sites for tryptic cleavage at the cytoplasmic surface of the membrane indicated. The findings described above are fully compatible with this model, where residues 207 to 271 and 450 to 492 are predicted to be exposed on the cytoplasmic surface of the membrane. All the potential sites of tryptic cleavage in the central hydrophilic region of the sequence were found to be cleaved, confirming the cytoplasmic location of this part of the protein (Fig. 8). Similarly, most of the potential sites of cleavage in the C-terminal hydrophilic region of the protein were cleaved by trypsin. The lack of cleavage at Lys-451 may have resulted from the proximity of this residue to the phospholipid headgroups in the membrane.

Although there are potential sites of tryptic cleavage at Lys-6, Lys-7 and Arg-11, no cleavage at these residues was detected. This finding indicates that the N-terminal region of the protein is not readily accessible to proteases in the native protein. This conclusion is supported by the finding that antibodies raised against a synthetic peptide corresponding to residues 1–15 cannot bind to the protein in its native conformation, but do recognise the denatured protein [12]. These antibodies do recognise the glycosylated tryptic fragment of the transporter on Western blots, confirming that it retains at least some of the N-terminal 15 amino acid residues of the protein.

The extramembranous loops predicted to connect helices 2 and 3, 4 and 5, and 8 and 9 (numbered from the N-terminus of the protein, according to the model proposed in Ref. 1) on the cytoplasmic side of the membrane were not cleaved by trypsin under the conditions used, although they contain several potential sites of cleavage (Fig. 8). Lack of cleavage may stem from their not

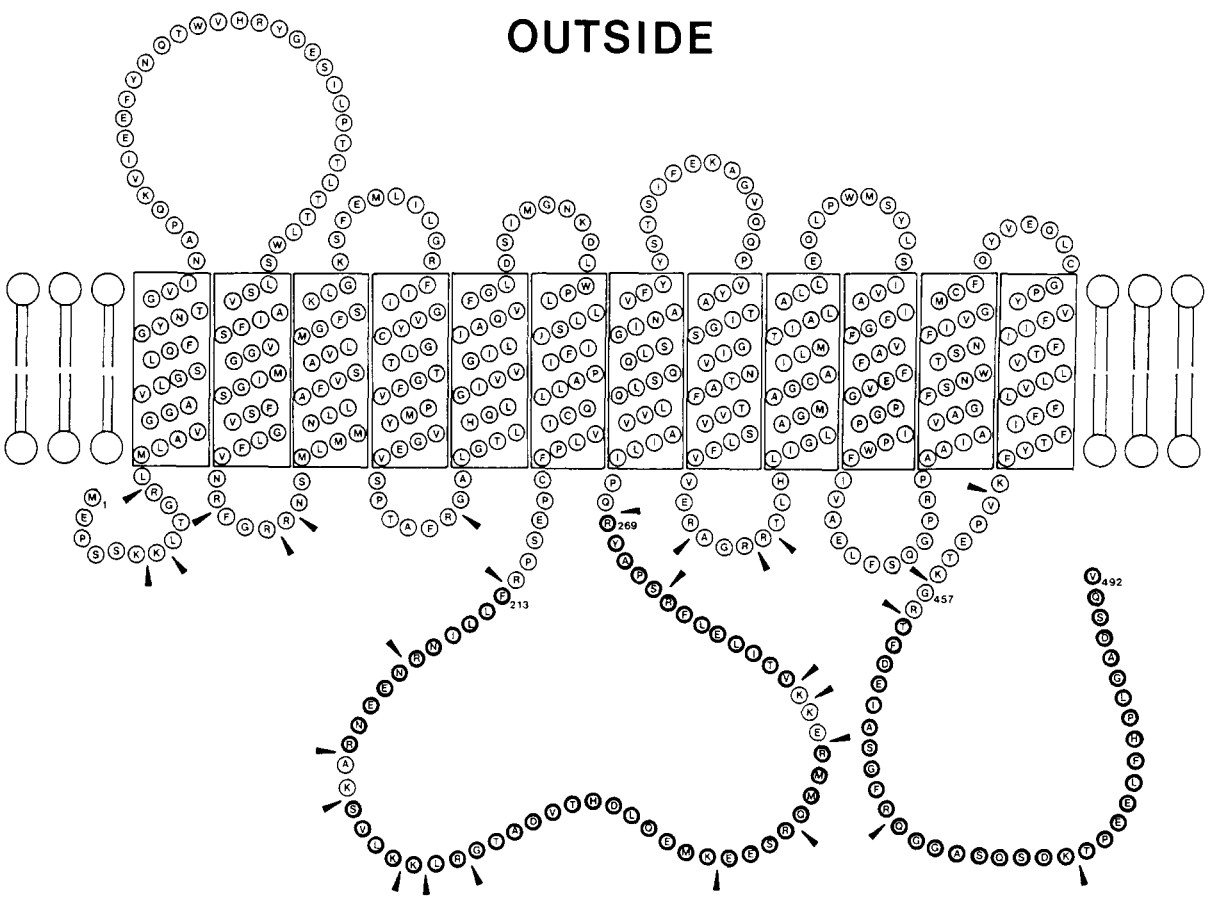

Fig. 8. A model for the arrangement of the glucose transporter polypeptide in the lipid bilayer. The model is based on that previously proposed [1]. Arrows indicate potential sites of tryptic cleavage on the cytoplasmic side of the membrane. Circles with a heavy outline indicate regions of the sequence identified in the form of small, soluble peptides in a tryptic digest of the native transport protein. Amino acids are identified by the single letter code.

extending far enough away from the phospholipid head groups to allow access to trypsin. We have found that if the digestion is performed in the presence of 0.1% Triton X-100, further fragmentation of the protein is obtained, indicating that at least some of these potential sites of tryptic cleavage are not intrinsically resistant to trypsin (Cairns, M.T. and Baldwin, S.A., unpublished observations).

In previous studies, we and others showed that the tryptic fragment of the transporter of apparent M_r 18000 contained the site(s) photolabelled by cytochalasin B [15,16]. It follows that this site(s) must lie between residues 270 and 456. We further showed that cleavage of the cytochalasin B-photolabelled transporter at its cysteine residues with 2-nitro-5-thiocyanobenzoic acid (NTCB) yielded predominantly an unlabelled, glycosylated fragment of apparent M_r 38000 and a labelled, non-glycosylated fragment of apparent M_r 15500 [15]. Preferential cleavage of the protein at Cys-347 would yield a glycosylated fragment of M_r 38000 comprising residues 1–346, and a non-glycosylated fragment of M_r 16000 corresponding to residues 347–492. This pattern of fragments could not be generated by cleavage at other cysteine residues. It follows that the site of cytochalasin B labelling must lie between residues 347 and 456.

In addition to the major labelled fragment of apparent M_r 15500, we also found small amounts of labelled material of lower M_r on SDS-polyacrylamide gels of the NTCB-cleaved transporter [15]. Recently Holman and Rees [28], in a study of the cleavage of the cytochalasin B-photolabelled

transporter in human erythrocyte membranes, established conditions under which a substantial yield of a labelled fragment of apparent M_r 5500 was produced by NTCB cleavage. They proposed that this fragment comprised residues 347–420 of the protein (predicted M_r 8007). They also showed that cleavage of the labelled protein with *N*-bromosuccinimide, which preferentially cleaves at tryptophan residues, yielded a labelled fragment of apparent M_r 3000. It was suggested that this fragment comprised residues 388 to 412. However, since *N*-bromosuccinimide cleaves at the C-terminal side of tryptophan, the fragment (if correctly identified) must in fact comprise residues 389 to 412 (predicted M_r 2549).

Deziel and colleagues [29] have proposed that photolabelling proceeds via photoactivation of an aromatic amino acid residue on the transporter, rather than by photoactivation of the cytochalasin B molecule itself. The segment of the protein identified above contains four aromatic residues which might represent the site of photolabelling by cytochalasin B (Phe-384, Phe-395, Phe-409 and Trp-412). Only Trp-412 is conserved in the sequence of the homologous arabinose transport protein from *Escherichia coli* [30], which can also be photolabelled by cytochalasin B (Smith, G., Cairns, M.T., Maiden, M.C.J., Henderson, P.J.F. and Baldwin, S.A., unpublished observations). It is therefore possible that this tryptophan residue is the site of labelling, although its definitive identification will of course require the isolation and characterisation of the labelled fragment itself.

FTIR spectroscopy of the trypsin-digested transporter indicated that the parts of the protein cleaved by this enzyme contain α-helical structure. Chin et al. [9] have previously predicted the presence of a significant portion of α-helical structure in the extramembranous domain of the protein, from a comparison of the α-helical content of the intact transporter, determined by circular dichroism, with the amount of α-helix required to form 12 membrane-spanning segments. However, our findings represent the first direct evidence for extramembranous α-helix. In support of these findings, the secondary structure prediction methods of Chou and Fasman [31] and of Garnier et al. [32] predict that the central hydrophilic region of the transporter sequence is predominantly α-helical, with residues 220–232 and 235–265 probably forming helices. The C-terminal hydrophilic segment of the protein (residues 451–492) is also predicted to contain short stretches of α-helix, together with β-turn and random coil structure (data not shown). Although the application of such methods to the prediction of the secondary structures within the hydrophobic domains of membrane proteins has been shown to be invalid, they have met with some success when applied to the extramembranous parts of such proteins [33]. We therefore feel that it is not unreasonable to apply them to the analysis of the very hydrophilic cytoplasmic domain of the glucose transporter.

We have previously interpreted the absorption bands found in the FTIR spectrum of the native transporter at approximately 1630 cm^{-1} and 1640 cm^{-1} as indicative of the presence of β-structure in this protein [10]. The presence of additional bands at about 1680 cm^{-1} and 1690 cm^{-1} suggests that at least some of the β-strands form anti-parallel chains [34], although absorptions near these frequencies have also been reported for β-turns [35,36] and amino acid side chains [36]. All four bands were seen in increased intensity relative to the α-helix/random coil band at 1657 cm^{-1} in the spectrum of the trypsin-digested transporter in H_2O (Fig. 4). It is therefore likely that the two large tryptic fragments of the transporter comprising residues 1–212 and 270–456 retain the β-structure present in the native transporter. In addition, the retention of a substantial proportion of the α-helical structure was indicated by the spectra, as would be predicted from the model that we have proposed for the transporter structure [1]. The fact that the membrane-bound tryptic fragments of the transporter are still able to bind both cytochalasin B and D-glucose, with apparent affinities similar to those seen with the intact protein, suggests that the conformation of the fragments is not drastically affected by tryptic digestion.

Although the membrane-spanning segments of integral membrane proteins are often presumed to be α-helical, there are some transmembrane proteins, such as porin, that are considered to be dominated by β-sheet structure [37,38]. Some of the membrane-spanning segments of the glucose transporter may therefore have a β-sheet confor-

mation. However, the model that has been proposed for the arrangement of the transporter polypeptide in the membrane predicts that the large tryptic fragments of the protein contain several extramembranous loops connecting the membrane-spanning segments [1]. These loops, rather than the intramembranous parts of the protein, may give rise to the β-structure bands seen in the FTIR spectra. For example, the postulated extracellular loop comprising residues 34–66, which bears the oligosaccharide chain of the transporter, is predicted by both the Chou and Fasman [31] and the Robson [32] algorithms to contain β-structure (data not shown).

In an attempt to differentiate between the two possible locations of the β-structure in the transporter discussed above, we subjected the transporter to exhaustive treatment with papain, which has a broad substrate specificity. Cleavage with papain for three days converted most of the transporter preparation to material that migrated near the tracking dye on 12% acrylamide gel (Fig. 6d). On gradient acrylamide gels, a substantial proportion of this material was found to migrate as a diffuse band between an apparent M_r 3000–4000 and the tracking dye (Fig. 6f). A fragment of the transporter derived from a single membrane-spanning α-helix would be predicted to have an M_r of 2000–3000, whereas a fragment containing a pair of linked helices would be predicted to have an M_r of at least 5000. It is therefore apparent that most of the predicted extramembranous loops of the transporter polypeptide were cleaved during prolonged exposure to papain. However, the four bands in the FTIR spectrum at about 1630, 1640, 1680 and 1690 cm^{-1} that we have attributed to β-sheet structure remained in the spectrum of the papain-digested protein (Fig. 7). These bands are therefore likely to arise from parts of the transporter sequence that are buried in the lipid bilayer, and which may be membrane-spanning.

In summary, we have shown that several features of the model previously proposed for the arrangement of the glucose transporter polypeptide in the membrane are correct. These include the presence of large extramembranous segments near the middle and at the C-terminus of the sequence. The membrane-spanning portions of the sequence appear to be largely α-helical, as predicted, but evidence has been obtained for the additional presence of some β-structure in this part of the protein. The large hydrophilic segments of the protein which are exposed at the cytoplasmic surface of the membrane do not appear to be essential for substrate or inhibitor binding, since the trypsin-digested protein is still able to bind both cytochalasin B and D-glucose. However, they are essential for transport itself, since tryptic digestion of inside-out erythrocyte membrane vesicles destroys their ability to carry out stereospecific hexose transport [39].

Acknowledgements

This work was supported in part by project grants from the Medical Research Council (to S.A.B.) and from the Science and Engineering Research Council (to S.A.B. and D.C.). We are also grateful to the Medical Research Council for the Imperial College Protein Chemistry Facility (program grant to H.R.M.), and to the Central Research Fund of the University of London for an equipment grant. J.A. was supported by a Research Fellowship from the Wellcome Trust. We are indebted to Dr. M.C.J. Maiden and Dr. S.J. Perkins for assistance with secondary structure prediction algorithms.

References

1 Mueckler, M., Caruso, C., Baldwin, S.A., Panico, M., Blench, I., Morris, H.R., Lienhard, G.E., Allard, W.J. and Lodish, H.F. (1985) Science 229, 941–945
2 Kasahara, M. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7384–7390
3 Baldwin, S.A., Baldwin, J.M. and Lienhard, G.E. (1982) Biochemistry 21, 3836–3842
4 Wheeler, T.J. and Hinkle, P.C. (1981) J. Biol. Chem. 256, 8907–8914
5 Connolly, T.J., Carruthers, A. and Melchior, D.L. (1985) J. Biol. Chem. 260, 2617–2620
6 Sogin, D.C. and Hinkle, P.C. (1978) J. Supramol. Struct. 8, 447–453
7 Gorga, F.R., Baldwin, S.A. and Lienhard, G.E. (1979) Biochem. Biophys. Res. Commun. 91, 955–961
8 Lee, D.C., Elliot, D.A., Baldwin, S.A. and Chapman, D. (1985) Biochem. Soc. Trans. 13, 684–685
9 Chin, J.J., Jung, E.K.Y. and Jung, C.Y. (1986) J. Biol. Chem. 261, 7101–7104
10 Alvarez, J., Lee, D.C., Baldwin, S.A. and Chapman, D. (1987) J. Biol. Chem. 262, 3502–3509

11 Birnbaum, M.J., Haspel, H.C. and Rosen, O.M. (1986) Proc. Natl. Acad. Sci. USA 83, 5784–5788
12 Davies, A., Meeran, K., Cairns, M.T. and Baldwin, S.A. (1987) J. Biol. Chem. 262, 9347–9352
13 Lienhard, G.E., Crabb, J.H. and Ransome, K.J. (1984) Biochim. Biophys. Acta 769, 404–410
14 Shanahan, M.F. and D'Artel-Ellis, J. (1984) J. Biol. Chem. 259, 13878–13884
15 Cairns, M.T., Elliot, D.A., Scudder, P.R. and Baldwin, S.A. (1984) Biochem. J. 221, 179–188
16 Deziel, M.R. and Rothstein, A. (1984) Biochim. Biophys. Acta 776, 10–20
17 Gorga, F.R. and Lienhard, G.E. (1981) Biochemistry 20, 5108–5113
18 Zoccoli, M.A., Baldwin, S.A. and Lienhard, G.E. (1978) J. Biol. Chem. 253, 6923–6930
19 Munson, P.J. and Rodbard, D. (1980) Anal. Biochem. 107, 220–239
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
21 Laemmli, U.K. (1970) Nature 227, 680–685
22 Hashimoto, F., Horigome, T., Kanbayashi, M., Yoshida, K. and Sugano, H. (1983) Anal. Biochem. 129, 192–199
23 Morris, H.R., Panico, M., Barber, M., Bordoli, R.S., Sedgwick, R.D. and Tyler, A.N. (1981) Biochem. Biophys. Res. Commun. 101, 623–631
24 Morris, H.R., Panico, M. and Taylor, G.W. (1983) Biochem. Biophys. Res. Commun. 117, 299–305
25 Gray, W.R. (1972) Methods Enzymol. 25, 121–138
26 Kauppinen, J.K., Moffat, D.J., Mantsch, H.H. and Cameron, D.G. (1981) Appl. Spectrosc. 35, 271–276
27 Lee, D.C., Hayward, J.A., Restall, C.J. and Chapman, D. (1985) Biochemistry 24, 4364–4373
28 Holman, G.D. and Rees, W.D. (1987) Biochim. Biophys. Acta 897, 395–405
29 Deziel, M., Pegg, W., Mack, E., Rothstein, A. and Klip, A. (1984) Biochim. Biophys. Acta 772, 403–406
30 Maiden, C.J., Davis, E.O., Baldwin, S.A., Moore, D.C.M. and Henderson, P.J.F. (1987) Nature 325, 641–643
31 Chou, P.Y. and Fasman, G.D. (1978) Advances Enzymol. 47, 45–148
32 Garnier, J., Osguthorpe, D.J. and Robson, B. (1978) J. Mol. Biol. 120, 97–120
33 Wallace, B.A., Cascio, M. and Mielke, D.L. (1986) Proc. Natl. Acad. Sci. USA 83, 9423–9427
34 Susi, H. and Byler, D.M. (1983) Biochem. Biophys. Res. Commun. 115, 391–397
35 Bandekar, J. and Krimm, S. (1980) Biopolymers 19, 31–36
36 Olinger, J.M., Hill, D.M., Jacobsen, R.J. and Brody, R.S. (1986) Biochim. Biophys. Acta 869, 89–98
37 Kleffel, B., Garavito, R.M., Baumeister, W. and Rosenbusch, J.P. (1985) EMBO J. 4, 1589–1592
38 Rosenbusch, J.P. (1974) J. Biol. Chem. 249, 8019–8029
39 Baldwin, J.M., Lienhard, G.E. and Baldwin, S.A. (1980) Biochim. Biophys. Acta 599, 699–714