The role of cysteine residues in glucose-transporter-GLUT1-mediated transport and transport inhibition

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The role of cysteine residues in transport function of the glucose transporter GLUT1 was investigated by a mutagenesis–expression strategy. Each of the six cysteine residues was individually replaced by site-directed mutagenesis. Expression of the heterologous wild-type or mutant glucose transporters and transport measurements at two hexose concentrations (50 μM and 5 mM) were undertaken in *Xenopus* oocytes. The catalytic activity of GLUT1 was retained, despite substitution of each single cysteine residue, which indicated that no individual residue is essential for hexose transport. This finding questions the involvement of oligomerization or intramolecular stabilization by a single disulphide bond as a prerequisite for transporter activation under basal conditions. Application of the impermeant mercurial thiol-group-reactive reagent *p*-chloromercuribenzenesulphonate (pCMBS) to the external or internal surface of plasma membrane demonstrated that cysteine-429, within the sixth external loop, and cysteine-207, at the beginning of the large intracellular loop which connects transmembrane segments 6 and 7, are the residues which are involved in transport inhibition by impermeant thiol-group-reactive reagents from either side of the cell. These data support the predicted membrane topology of the transport protein by transport measurements. If residues other than the cysteines at positions 429 or 207 are exposed to either side of the plasma membrane by conformational changes, they do not contribute to the transport inhibition by pCMBS. Application of pCMBS to one side of the plasma membrane also inhibited transport from the opposite direction, most likely due to the hindrance of sugar-induced interconversion of transporter conformation.

INTRODUCTION

Six facilitative glucose transporters have been cloned and these are named GLUT1–GLUT5 and GLUT7 according to the chronology of cloning their genes. An almost identical topological organization with 12 transmembrane α-helices has been predicted (Mueckler et al., 1985) and 42–65% amino acid identity exists between GLUT1 compared with GLUTs 2–5 (Gould and Bell, 1990; Bell et al., 1990). Derived from the nucleotide sequence of the cDNA, GLUT1 is known to possess six cysteine residues (i.e. C^{133}, C^{201}, C^{207}, C^{347}, C^{421} and C^{429}). Cysteine residues in positions 207 and 347 are conserved in GLUTs 1–4, but excluded from GLUT5, which is the least similar member of the family and is unique due to its specificity for fructose (Burant et al., 1992). On the basis of the putative two-dimensional model of GLUT1, C^{133} and C^{347} are harboured within the centre of transmembrane segments 4 and 9, whereas C^{201} and C^{421} are located towards the end of helices 6 and 11 respectively. C^{207} is exposed to the cytoplasmic side, whereas C^{429} is accessible from the outside of the cell membrane.

Recent reviews (e.g. Walmsley, 1988; Carruthers, 1990; Silverman, 1991) have discussed the importance of cysteine residues for glucose transport. In this respect, cysteine residues may be involved in oligomerization, as proposed by Hebert and Carruthers (1992), or may be important for stabilization of a local tertiary structure by disulphide bonds. Moreover, thiol-group-reactive reagents are known to inhibit glucose transport, probably by impairment of the interconversion of transporter conformation. C^{207} and C^{429}, upon reacting with impermeant inhibitors from inside or outside of the cell, are the most likely candidates for stabilizing the inward- or outward-facing conformation of the transporter. Since the exact boundaries of the transmembrane segments are difficult to predict (Baldwin, 1992), we used *p*-chloromercuribenzenesulphonate (pCMBS) to demonstrate that C^{429} is the only cysteine residue accessible for impermeant thiol-group-reactive reagents from the cell surface (Wellner et al., 1992). This agrees with the putative topology of GLUT1 proposed by Mueckler et al. (1985).

The present study was undertaken by carrying out site-directed mutagenesis and expression of wild-type or mutant glucose transporter GLUT1 in *Xenopus* oocytes to address the following questions. Firstly, are single cysteine residues essential for basal activity of the transporter, as must be expected if oligomerization by disulphide-bond formation is a precondition for catalytic activation? Secondly, which of the two outward-facing (C^{421}, C^{429}) or two inward-facing (C^{201}, C^{207}) cysteine residues are involved in transport inhibition from outside or inside of the cell? Substitution of these residues followed by application of impermeant thiol-group-reactive reagents like pCMBS would definitely answer which cysteine residue is involved in glucose transport inhibition from either side of the plasma membrane. Thirdly, does application of the impermeant transport inhibitor pCMBS from the internal or external side of the plasma membrane impair glucose transport from the opposite direction (supporting the notion that the transporter is locked into either conformation)?

MATERIALS AND METHODS

Engineering of mutants and *in vitro* synthesis of cRNA

A 2.4 kbp fragment derived from pSPGT (Mueckler and Lodish, 1986) was subcloned into pBluescript SK (Stratagene, Heidel-

Abbreviations used: pCMBS, *p*-chloromercuribenzenesulphonate; MBS, modified Barth's solution; CB, cytochalasin B; NEM, *N*-ethylmaleimide; DTT, dithiothreitol.

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berg, Germany). Site-directed mutagenesis was performed by the procedure of Kunkel et al. (1987) using a Bio-Rad kit (Bio-Rad, München, Germany). The resulting changes of the mutagenic oligonucleotides and the amino acids are as follows:

Amino acid no.	Nucleotide change	Amino acid change
C^{133}	5′TGC3′→5′AGC3′	Cys→Ser
C^{201}	5′TGC3′→5′GGC3′	Cys→Gly
C^{207}	5′TGC3′→5′AGC3′	Cys→Ser
C^{347}	5′TGC3′→5′AGT3′	Cys→Ser
C^{421}	5′TGC3′→5′CGC3′	Cys→Arg
C^{429}	5′TGT3′→5′TCT3′	Cys→Ser

Mutagenesis was confirmed by DNA sequence analysis. Wild-type and mutant cDNAs were subcloned into the oocyte expression vector pSP64T (Krieg and Melton, 1984). After *in vitro* transcription, proof of full-length cRNA and quantification of newly synthesized cRNA were performed as described previously (Garcia et al., 1992). The final concentration of all cRNA preparations was adjusted to 0.5 mg/ml by counting the incorporated [^{35}S]thio-UTP and by comparison of the wild-type and mutant cRNAs on a denaturing agarose gel, also including 28 S and 18 S rRNA as standards (Pharmacia, Freiburg, Germany).

Xenopus oocytes as an expression system, microinjection of pCMBS, and transport measurement

Collection, defolliculation and culture of *Xenopus* oocytes were conducted routinely as described previously (Keller et al., 1989; Garcia et al., 1992). 2-Deoxy-D-[^{3}H]glucose or 3-*O*-methyl-D-[^{3}H]glucose uptake assays were performed for comparison of hexose transport into cells between wild-type and mutant GLUT1-expressing oocytes. In brief, after 30 min incubation of oocytes in 0.5 ml of ice-cold modified Barth's solution (MBS) containing ^{3}H-labelled 2-deoxy-D-glucose (50 μM or 5 mM; 1 or 5 μCi/0.5 ml) or 3-*O*-methyl-D-glucose (5 mM; 5 μCi/0.5 ml), the oocytes were washed three times with MBS containing 0.1 mM phloretin, and then dissolved in 1% SDS before the radioactivity was counted. 3-*O*-Methylglucose efflux measurements were conducted after equilibrating oocytes with ^{3}H-labelled 3-*O*-methyl-D-glucose (50 μM) for 12 h, followed by twice rinsing the cells in a Petri dish containing 10 ml of ice-cold MBS; then 10 oocytes per group were transferred into 0.1 ml of MBS which had been adjusted previously to room temperature. After 10 and 20 min of gently shaking the oocytes, aliquots of 10 and 50 μl of the medium were removed for quantification of radioactivity by liquid-scintillation spectrophotometry. In order to determine which cysteine residue is involved in transport inhibition by impermeant thiol-group-reactive reagents from outside or inside of the cell, *Xenopus* oocytes were either incubated with pCMBS (0.5 mM; see Batt et al., 1976) or were microinjected with 75 nl of pCMBS to adjust its concentration to 3 mM. Since it is possible that minute amounts of pCMBS may be accidentally released into the medium during the injection procedure and then may bind to exofacial cysteine residues, oocytes were rinsed immediately thereafter with a large volume of fresh MBS. After 60 min, 2-deoxy-D-glucose uptake measurements were carried out.

The mutant oligonucleotides were purchased from Molbiol (Berlin, Germany), the radiolabelled glucose analogues (2-deoxy-D-[2,6-^{3}H]glucose and 3-*O*-methyl-D-[1-^{3}H]glucose) from Amersham-Buchler (Braunschweig, Germany). The Sequenase version 2 DNA sequencing kit was from USB (Cleveland, OH, U.S.A.), phloretin and *p*-chloromercuribenzene sulphonic acid (pCMBS) were received from Sigma Chemicals (St. Louis, MO, U.S.A.). All other substances used were of the highest grade. *Xenopus laevis* frogs were obtained from Xenopus Laborzucht (H. Kähler, Hamburg, Germany). Statistical evaluation was done using Student's *t* test for paired values (conducted with StatView version 4 for the Macintosh, Abacus Concepts, Inc., Berkeley, CA, U.S.A.).

RESULTS

Derived from the cDNA, GLUT1 is known to possess six cysteine residues. Table 1 demonstrates that uptake rates which have been determined at 50 μM or 5 mM (K_m for net influx 6.9 mM; Gould and Holman, 1993) concentration of 2-deoxy-D-glucose were comparable between wild-type and mutant glucose transporter-expressing oocytes. Substitution of each single cysteine amino acid indicated that no individual residue is an absolute requirement for maintaining the catalytic activity of GLUT1. In addition, replacement of both inward-facing cysteine residues within the same transporter (mutant C^{201}G-C^{207}S) also retained transport activity. As oocytes from different experiments do not necessarily express the same amount of glucose-transporter proteins, a paired comparison from independent experiments was conducted for each mutant by setting wild-type transporter activity to 100%. With 28%, maximum deviation from wild-type-mediated uptake was reasonably small and most likely reflects minor variations of injected amounts of cRNA that seemed to be unavoidable, despite careful comparison and

Table 1 Substitution of single cysteine residues does not influence basal 2-deoxy-D-glucose uptake

Xenopus oocytes were injected with wild-type or mutant GLUT1 cRNAs. After 3 days, ^{3}H-labelled 2-deoxy-D-glucose (2-DOG) (50 μM or 5 mM) uptake rates of single oocytes were determined in independent experiments for each cysteine mutant. Groups of 8–20 oocytes were included in the comparison using Student's *t* test for paired values. Results are mean percentages related to 2-deoxy-D-glucose uptake by wild-type GLUT1 (= 100%).

	Percentage of wild-type GLUT1 transport activity			
[2-DOG]...	50 μM		5 mM	
Mutant	Mean	*P* value	Mean	*P* value
C^{133}S	101	0.87	89	0.06
	114	0.05		
C^{201}G	88	0.12	91	0.26
	100	0.73		
C^{207}S	93	0.49	102	0.85
	104	0.97		
C^{201}G-G^{207}S	110	0.28	92	0.06
	122	0.05	119	0.17
C^{347}S	93	0.58	90	0.24
	124	0.66		
C^{421}R	97	0.31	105	0.46
	107	0.93		
C^{429}S	82	0.23	100	0.95
	85	0.29	101	0.83
	85	0.05		

Table 2 Effect of external pCMBS on 2-deoxy-D-glucose uptake

In two independent experiments, uptake of ^{3}H-labelled 2-deoxy-D-glucose at 5 mM was determined in *Xenopus* oocytes expressing the mutants $C^{421}R$ or $C^{429}S$. Before uptake measurements oocytes were exposed to 0.5 mM pCMBS for 1 h at room temperature. Data are from 14–20 oocytes per group. Values are means ± S.E.M.

Mutant	pCMBS...	Uptake rate (nmol/30 min per oocyte) −	+	Activity remaining (%)
Expt. 1				
$C^{421}R$		2.81 ± 0.11	0.80 ± 0.20	28
$C^{429}S$		3.25 ± 0.16	3.14 ± 0.22	97
Expt. 2				
$C^{421}R$		2.29 ± 0.21	0.83 ± 0.07	36
$C^{429}S$		1.99 ± 0.14	2.16 ± 0.12	109

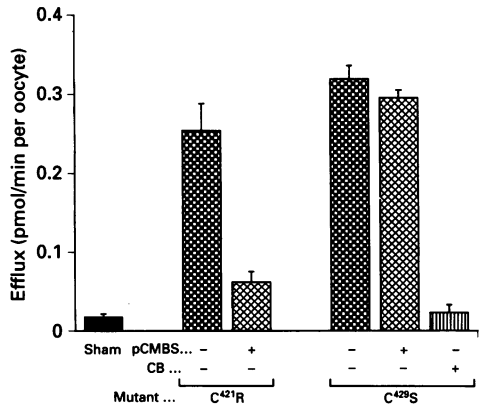

Figure 1 Effect of external pCMBS on 3-*O*-methyl-D-glucose efflux

Xenopus oocytes expressing the mutant glucose transporters after substitution of cysteine at position 421 ($C^{421}R$) or 429 ($C^{429}S$) were equilibrated with ^{3}H-labelled 3-*O*-methyl-D-glucose (50 μM) for 12 h. Oocytes were then exposed to pCMBS (0.5 mM) for 1 h before 3-*O*-methyl-D-glucose zero-*trans* efflux measurements were performed. Transport mediated by $C^{429}S$ containing GLUT1 was inhibited by CB (20 μM). Data are from three assays per mutant, each including 10 oocytes in 0.1 ml of MBS (for details, see the Materials and methods section). Results are means ± S.E.M.

quantification after its *in vitro* transcription (see the Materials and methods section).

According to the hypothetical topology of GLUT1, C^{421}, at the end of helix 11, and C^{429}, within the external loop connecting the membrane-spanning segments 11 and 12, are considered to be the most likely candidates for exofacial thiol-group-reactive reagents. It is known from our previous data obtained at the 'low' (50 μM) substrate concentration that C^{429} is the only cysteine of GLUT1 which exposes thiol groups to the outside of the plasma membrane, where they can react with extracellular impermeant substrates, resulting in inhibition of 2-deoxy-D-glucose uptake (Wellner et al., 1992). In order to demonstrate that this is also the case at higher transport rates, 2-deoxy-D-glucose at 5 mM concentration was used for uptake measurements. The data shown in Table 2 confirmed our previous data that after replacement of C^{429} external pCMBS is unable to inhibit 2-deoxy-D-glucose uptake. This shows that residues other

Table 3 Effect of internal pCMBS on 2-deoxy-D-glucose (2-DOG) and 3-*O*-methyl-D-glucose (3-OMG) uptake

In three independent experiments, *Xenopus* oocytes were injected with cRNAs encoding the cysteine mutants at position 201 ($C^{201}G$) or 207 ($C^{207}S$). After 3 days pCMBS was microinjected into oocytes, increasing the intracellular concentration to 3 mM. At 1 h after pCMBS application, ^{3}H-labelled 2-deoxy-D-glucose (50 μM) or 3-*O*-methyl-D-glucose (5 mM) uptake measurements were conducted in 12–20 single oocytes per group. Results are means ± S.E.M.

Mutant	pCMBS...	Uptake rate (pmol of 2-DOG/30 min per oocyte) −	+	Activity remaining (%)
Expt. 1				
$C^{201}G$		62.5 ± 2.5	36.4 ± 2.5	58
$C^{207}S$		60.1 ± 3.1	56.5 ± 3.3	94
Expt. 2				
$C^{201}G$		43.1 ± 2.1	18.1 ± 1.2	42
$C^{207}S$		46.9 ± 3.1	40.5 ± 4.3	86

Mutant	pCMBS...	Uptake rate (nmol of 3-OMG/30 min per oocyte) −	+	Activity remaining (%)
Expt. 3				
Wild-type		1.31 ± 0.05	0.88 ± 0.04	67
$C^{201}G$		1.44 ± 0.05	1.02 ± 0.05	71
$C^{207}S$		1.22 ± 0.04	1.12 ± 0.04	92

than C^{429} do not contribute to transport inhibition on reaction with pCMBS. The remaining transport activity after pCMBS application was 28 % at 50 μM, compared with 32 % (average of two independent experiments) at 5 mM, 2-deoxy-D-glucose.

The question remains to be answered of whether reaction of exofacial thiol groups of C^{429} would also have an impact on the efflux of glucose from glucose-preloaded oocytes by locking the transporter into an outward-facing conformation, and if this can be prevented by replacement of this particular cysteine residue. Figure 1 shows that external pCMBS was able to effectively inhibit efflux of 3-*O*-methyl-D-glucose and that substitution of C^{429} rendered the transporter protein unresponsive to external pCMBS. In addition, it was evident that, because of restricted permeability, pCMBS does not penetrate the plasma membrane to any great extent. After 1 h of incubation, there was no inhibitory reaction with inward-facing cysteine residues (i.e. C^{201} and C^{207}) or cysteine residues harboured in the middle of putative transmembrane segments (i.e. C^{133} and C^{347}). In order to estimate the amount of non-specific efflux, cytochalasin B (CB) was included in the transport assay of the C^{429} mutant; the remaining efflux of 3-*O*-methyl-D-glucose into the medium was similar to that of water-injected *Xenopus* oocytes ('Sham'). As we know from previous experiments (Wellner et al., 1992) that substitution of C^{421} strikingly reduces CB-mediated inhibition of glucose transport, CB was not included in this assay.

Injection of pCMBS into oocytes expressing wild-type, $C^{201}G$ or $C^{207}S$ mutants allowed us to localize those cysteine residues which are accessible from the cytoplasmic side of the plasma membrane, resulting in inhibition of transport. The amount of intracellular pCMBS was adjusted to 3.0 mM to inhibit uptake of 2-deoxy-D-glucose or 3-*O*-methyl-D-glucose. The data of Table 3 demonstrate that, if C^{207} is accessible, uptake rates were then

decreased by 50% at 50 μM 2-deoxy-D-glucose (average of two independent experiments) or by 31% at 5 mM 3-*O*-methyl-D-glucose (average of two assays). 3-*O*-Methyl-D-glucose was chosen instead of 2-deoxy-D-glucose when the higher concentration was used, because we could not rule out the possibility that pCMBS may affect hexokinase activity, rendering phosphorylation the rate-limiting step of hexose uptake. In most experiments, decreasing the intracellular concentration of pCMBS to less than 1 mM nullified the inhibitory effect, whereas levels higher than 3 mM increased the CB-insensitive portion of glucose transport several-fold. Therefore, only at a very narrow range of intracellular concentrations did injection of pCMBS appear to be a useful approach to determine which cysteine residue is the most likely candidate to react with pCMBS and cause inhibition of transport. The data support the proposed two-dimensional structure of GLUT1 within the plasma membrane and also document that, in agreement with results from Figure 1, covalent binding of thiol-group-reactive reagents to cysteine residues from either side of the membrane inhibited glucose transport from the opposite direction. The direct approach by determining zero-*trans* efflux of 3-*O*-methyl-D-glucose from prelabelled and pCMBS-co-injected oocytes was avoided for reasons discussed below.

DISCUSSION

Recent studies have begun to address the role of single amino acids of the glucose transporter GLUT1 in transport function and binding of specific transport inhibitors. Site-directed mutagenesis in conjunction with heterologous expression in mammalian cells or *Xenopus* oocytes have so far been used to elucidate the functional consequence of changing (i) all tryptophan residues (Katagiri et al., 1991; Garcia et al., 1992), (ii) asparagine residues, including the invariant glycosylation site at Asn^{45} (Ishihara et al., 1991; Asano et al., 1991) and (iii) the glutamine residue at position 282 (Hashiramoto et al., 1992), which, among other amino acids of transmembrane segment 7, is a possible candidate for hydrogen-bonding with glucose. Until the transport protein has been crystallized, site-directed mutagenesis is the most efficient approach to study the functional significance of structural changes which are designed on the basis of the hypothetical two-dimensional orientation of the transporter within the plasma membrane. In our experience (Garcia et al., 1992), *Xenopus* oocytes have proved quite satisfactory as an expression system to test the function of wild-type or mutated glucose transporters. A further argument is that impermeant thiol-group-reactive reagents are able to react with endofacial cysteine residues of the glucose transporter upon co-injection without the need to permeabilize cells. Since the level of expression of heterologous proteins depends on various conditions, in particular the amount of injected nucleic acid or the varying quality of oocytes of individual frogs [reviewed by Keller and Mueckler (1990)], comparison between independent experiments was performed on a percentage basis (see Table 1).

The importance of cysteine residues for transporter function can be deduced theoretically from their possible involvement in oligomerization, stabilization of local tertiary structures, and transport inhibition by mercury-containing reagents. If such a substance is impermeant, its application can be instrumental to study the as yet speculative orientation of the glucose transporter within the plasma membrane. Important results were obtained from covalent modification studies by Deziel et al. (1985) using *N*-ethylmaleimide (NEM) as a thiol-group-reactive reagent that was incorporated into the purified or intact erythrocyte glucose transporter. Their data indicate that incorporation of NEM into a tryptic fragment of 19 kDa could be prevented by prior application of the impermeant pCMBS to intact erythrocytes. This fragment contains the C-terminal end of the transporter and covers roughly two-fifths of the protein, including three cysteine residues, i.e. C^{347}, C^{421} and C^{429}. Cysteine residues that are assumed to be also exposed by sugar-induced conformational changes are residues at position 347 or 421 [reviewed by Walmsley (1988) and Silverman (1991)]. C^{347}, located towards the middle of helix 9, seemed more likely to be involved, since fluorescence studies of the chloronitrobenzoxazole-bound transporter point to a rather hydrophobic region (Rampal and Jung, 1987). However, our transport studies after site-directed mutagenesis indicate that, except for the cysteine at position 429, no other cysteine residue contributes to inhibition of hexose influx if exposed to the outside of the membrane by conformational changes. When C^{429} was replaced, application of pCMBS failed to inhibit bidirectional transmembrane hexose movement, as proved by 2-deoxy-D-glucose influx (Wellner et al., 1992; Table 2) or 3-*O*-methyl-D-glucose efflux (see Figure 1). 3-*O*-Methyl-D-glucose efflux was slightly more inhibited by pCMBS than 2-deoxy-D-glucose influx, the difference appearing to be too small to claim asymmetry of transport kinetics, as previously described in erythrocytes by Batt et al. (1976).

There is still controversy whether a monomeric or an oligomeric structure determines the catalytic activity of GLUT1 (Pessino et al., 1991; Burant and Bell, 1992; Hebert and Carruthers, 1992). Even if glucose transporter molecules exist as a homo- or hetero-multimer within the plasma membrane, it does not necessarily imply that oligomerization is essential for functional activity. The free thiol groups of cysteine residues or the consensus sequence of the leucine zipper motif within the N-terminal 85 amino acids, among other mechanisms, might be involved in oligomerization. Findings by Hebert and Carruthers (1992) suggest that, upon purification, depending on the presence or absence of dithiothreitol (DTT), GLUT1 is obtained as a dimer (plus DTT) or tetramer (minus DTT). According to their model, the monomeric subunit exposes sugar-binding sites to either the exofacial or endofacial surface of the plasma membrane, but mutually excludes the other direction; whereas subunits of the dimer or tetramer are able to isomerize independently, but co-operatively, between the two conformational states favouring either glucose influx or efflux. It is noteworthy that, depending on the redox state, the capacity of the red-cell GLUT1 to bind CB is altered with a higher number of binding sites available after reduction of the transporter. Our data show that substitution of each single cysteine residue within GLUT1 (see Table 1) has no influence on 2-deoxy-D-glucose transport in GLUT1-expressing *Xenopus* oocytes. This indicates that no individual residue is essential for transporter activation, thus questioning oligomerization by a single disulphide bond as a crucial step for transport activation. However, it is possible that transporter oligomerization involves several pairs of cysteine residues, occurs without functional consequences, or that a multimeric assembly takes place by means different from disulphide bonding. To address this question, the mutant $C^{201}G$-$C^{207}S$ was included in Table 1. However, further mutagenesis would be necessary to have more than one single cysteine residue replaced within the same transporter molecule. On the basis of the findings of Hebert and Carruthers (1992), a distinct redox state could theoretically lead to the predominant expression of an oligomeric form of the heterologously expressed glucose transporter, thereby resulting in lower glucose-transport rates. Although unlikely, it could be that amphibian plasma membranes differ from erythrocyte membranes in that a redox-dependent

oligomerization is less likely to occur. Burant and Bell (1992) used a very elegant approach using *Xenopus* oocytes co-expressing glucose transporter isoforms that are unique for their extremely distinct kinetic properties. Their data suggest a functional monomeric form and do not support oligomerization as being a prerequisite for functional activity.

Consistent with the widely favoured mechanism of facilitative glucose transport with mutually exclusive inward- and outward-facing conformations of the protein are the following studies. Substrates like maltose or phloretin accelerate transporter inactivation by substrates from outside of the cell (Krupka, 1985) upon binding to the exofacial surface of the transporter, whereas CB, which probably reacts with the inward-facing tryptophan residue W^{388} (Garcia et al., 1992), has the opposite effect. May and Beechem (1993) reported that CB protects the exofacial transporter from being labelled by 2-(4′-maleimidylanilino)-naphthalene-6-sulphonic acid. In addition, glucose analogues or the mannose derivative 2-*N*-(4-azidosalicoyl)-1,3-bis-(D-mannos-4′-yloxy)propyl-2-amine, which bind to the outward-facing form of the transporter, decrease thermolysin sensitivity. On the other hand, sugars or CB, which bind preferentially to the endofacial site of the transporter, caused an increase in sensitivity (Holman and Rees, 1987; King et al., 1991). It is noteworthy that CB blocked both effects, probably by stabilizing a structure of the protein that inhibits the interconversion of conformational states. Hindrance of conformational changes may also explain why application of the almost impermeant thiol-group-reactive reagent pCMBS to the exofacial surface of the transporter resulted in inhibition of 2-deoxy-D-glucose uptake (Wellner et al., 1992; Table 2) and also inhibited efflux of 3-*O*-methyl-D-glucose (Figure 1). In accordance with these results, reaction of micro-injected pCMBS with the endofacial surface of the transporter effectively inhibited zero-*trans* 2-deoxy-D-glucose uptake. These and the aforementioned data of others argue against an oligomer that is able to react with inhibitors like CB or pCMBS and binds and transports sugars at the same time. The simplest conclusion from our data is that stabilization or locking of an outward- or inward-facing conformation inhibits binding and transport from the opposite site which is consistent with the alternating-conformer model.

The effect of cytoplasmic pCMBS on transport was only tested by zero-*trans* 2-deoxy-D-glucose uptake, as we decided not to inject pCMBS into 3-*O*-methyl-D-glucose-prelabelled *Xenopus* oocytes in order to avoid microinjection into cells which are maintained in a radioactive equilibrating medium. The rationale for measuring glucose transport from the opposite side is presented in Figure 1, where external pCMBS inhibited 3-*O*-methyl-D-glucose efflux. It was anticipated that, in accordance with these data, glucose influx could be inhibited by cytoplasmic pCMBS, as subsequently was confirmed in three independent experiments (see Table 3). For reasons unknown, pCMBS was less effective from the cytoplasmic than the external side in inhibiting hexose transport from a direction which is opposite to the inhibitor. A further rise in intracellular concentration of pCMBS increased the portion of CB-resistant 2-deoxy-D-glucose uptake several-fold. This could be due to gross structural changes of GLUT1, causing an almost total loss of inhibition by CB, or due to plasma-membrane alterations leading to greater permeability for glucose. One reasonable explanation of lower intracellular effectiveness is that multiple intracellular cysteine-containing proteins compete for the mercury-containing reagent with the one thiol group accessible/mol of GLUT1. This, and the narrow range of effective pCMBS concentrations in particular, makes the intracellular application of pCMBS a less useful approach than applying it from outside of the cell when one intends to investigate which cysteine residues are involved in pCMBS-mediated transport inhibition upon exposure to either side of the plasma membrane. Thus it is impossible to compare the effectiveness of single cysteine residues located towards the exofacial or endofacial surface with respect to inhibition of glucose transport. However, regarding the putative membrane topology of GLUT1, injection of pCMBS into oocytes appeared to provide valuable information as to which cysteine can contribute to transport inhibition from the cytoplasmic side of the plasma membrane.

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