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The construction of a cysteine-less melibiose carrier from *E. coli*

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Abstract

The melibiose carrier of *E. coli* is a cation-sugar cotransport system. This membrane protein contains four cysteine residues and the transport function is inhibited by sulfhydryl reagents. In order to investigate the importance of the cysteines, we have constructed a set of four melibiose transporters each of which has one cysteine replaced with serine or valine. The sensitivity of this set of carriers to *N*-ethylmaleimide was tested and Cys364 was identified as the target of the reagent. In addition, we constructed a melibiose transporter in which all 4 cysteines were replaced with either serine (Cys110, Cys310, and Cys364) or valine (Cys235) and we found that, as expected, the resulting cysteine-less transporter was resistant to the action of *N*-ethylmaleimide. The cysteine-less melibiose carrier had no significant decrease in ability to accumulate melibiose with cotransported sodium ions or protons. Thus, none of the 4 cysteines are necessary for the function of the melibiose carrier. © 1997 Elsevier Science B.V.

Keywords: Melibiose carrier; Cysteine; Mutagenesis; (*E. coli*)

1. Introduction

The melibiose transporter of *E. coli* is an integral membrane protein that facilitates the transport and accumulation of α-galactosides or β-galactosides into the cell. This symporter is unusual in that it can utilize a variety of cations (H^+, Na^+, or Li^+), as the cotransported ion (see reviews [1–3]). The gene for the *E. coli* protein (*melB*) has been sequenced by Yazyu et al. [4] and the protein has been overexpressed and purified by Pourcher et al. [5]. The hydropathy profile as well as experiments with *phoA* fusions [6,7] provide information about the two-dimensional structure of the melibiose carrier and suggest that the carrier possesses 12 transmembrane α-helices. The reaction of inside-out vesicles with an antibody prepared against the carboxyl-terminus of the protein demonstrates that this end of the protein extends into the cytoplasm [8].

Mutant selections and site-directed mutagenesis have begun to provide data on the specific amino acids and domains of the melibiose carrier that are important for its function. The clustering of mutants with altered sugar and/or cation specificity within the first 4 proposed transmembrane helices, helix 7, helices 10 and 11, and the loop between helices 10

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and 11, strongly suggest a close interaction of these regions in the three-dimensional structure of the melibiose carrier. For example, the four aspartic acid residues (at positions 19, 55, 59, and 124) in membrane-spanning helices 1, 2, and 4 appear to be important for Na^+ cotransport [9,10] and Asn58 (helix 2) has been identified as important in Na^+ specificity [11,12]. Additional mutants with altered sugar and/or cation specificity had amino acid changes in transmembrane helices 1, 4, 7, 10, and 11 and in the cytoplasmic loop between helices 10 and 11 [13–15].

The study of naturally occurring cysteines or those re-introduced by site-directed mutagenesis into functional cysteine-less transporters has yielded information about the route of the substrate through a variety of bacterial membrane carriers. The experimental finding that the substrates of transporters prevent inhibition of transport by sulfhydryl reagents suggests that the targeted cysteines are in the path that the substrate takes through the carrier. The targeted cysteines could directly participate in the binding of the substrate during its transit or the modified cysteine could physically block access to the transport path [16–20]. In addition, the reaction of cysteines with hydrophilic, membrane impermeant sulfhydryl reagents presented to right-side-out or inside-out membranes and the resulting effect on transport function has provided further evidence for the identification of amino acids that line the channel through which the transporter's substrate moves [17–20].

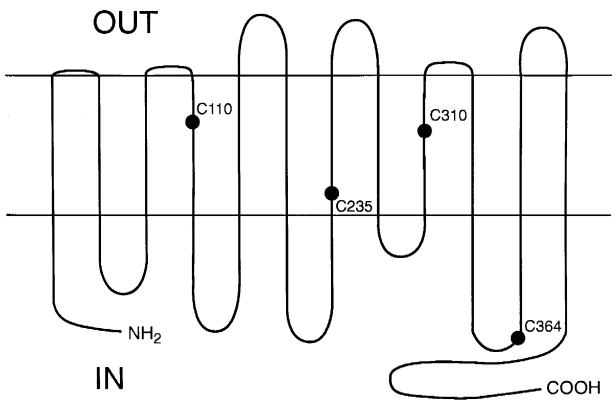

Fig. 1. Relative location of cysteines on the topographic map of the melibiose transporter. The 12 transmembrane segments that are predicted from hydropathy profiles and from alkaline phosphatase fusion proteins [6,7] are shown with the amino and carboxyl termini of the protein oriented to the inside of the cell [8]. The relative location of the 4 cysteines either in the transmembrane segments or in a cytoplasmic loop are as designated.

The melibiose transporter has 4 cysteines at residue number 110, 235, 310, and 364 as depicted schematically in Fig. 1. The first 3 cysteines are located in helices 4, 7, and 9 respectively, whereas Cys364 is located in the cytoplasmic loop between helices 10 and 11. Previously, the inhibition of melibiose transport by sulfhydryl reagents was shown by Cohn et al. [21] to be partially prevented by the presence of substrate. However, substrate binding (*p*-nitrophenyl-α-galactoside) was unaffected by sulfhydryl reagents [22]. In this communication we construct a set of 4 melibiose carriers each of which has one of the 4 cysteines changed to serine or valine. The effect that the elimination of a single cysteine has on transport and on inhibition of transport by *N*-ethylmaleimide is determined. A cysteine-less melibiose carrier is also constructed and its transport capabilities are analyzed.

2. Materials and methods

2.1. Reagents

Melibiose (6-*O*-α-D-galactopyranosyl-D-glucopyranoside), TMG (methyl-1-thio-β-D-galactopyranoside), and lactose were purchased from Sigma. [^{3}H]Melibiose was a generous gift from Dr. Gerard Leblanc of the Département de Biologie Cellulaire et Moléculaire du CEA, Villefranche-sur-mer, France. [^{14}C]TMG was purchased from Dupont-NEN. [α-^{33}P]dATP was from Andotek and [^{35}S]Protein A was purchased from Amersham. Bacteriological media were from Difco. *N*-ethylmaleimide (NEM) was from Sigma.

2.2. Bacterial strains and plasmids

E. coli DW1 (*lacI*$^+$ *ΔlacZY ΔmelAB*) [23] was used as the host strain for the plasmids when bacteria were grown for sugar transport assays, sugar-stimulated H^+ and Na^+ uptake, and immunoblot experiments. The plasmid, pKKMB [24], that contains the gene for the temperature resistant form of the melibiose carrier inserted into the vector pKK223-3

(Pharmacia Biotech) was used as the source of the *melB* gene into which the mutations were introduced.

2.3. Site-directed mutagenesis

The mutagenic oligonucleotides that are listed in Table 1 were used to replace the cysteine codons at positions 110, 310, and 364 with serine codons and the cysteine codon at position 235 by a valine codon in the temperature resistant *melB* gene of *E. coli* that is contained in the plasmid, pKKMB. Three of the individual substitutions for cysteine (at positions 110, 310, and 364) were constructed by the method of Taylor et al. [25,26] in an M13 derivative that contained the *melB* gene from pKKMB. The cysteine-less melibiose carrier was constructed with the Muta-Gene M13 in vitro Mutagenesis Kit (Bio-Rad) using the M13 derivative just described. The Cys235 to valine substitution was constructed with the Chameleon Double-stranded Site-directed Mutagenesis Kit (Stratagene).

2.4. DNA sequencing

Double-stranded plasmid DNA was isolated with a QIAprep Spin Miniprep Kit (Qiagen). DNA was sequenced with the AmpliCycle Sequencing Kit (Perkin Elmer).

2.5. Immunodetection of the melibiose carrier in bacterial cells

The method of Lolkema et al. [27] was used to detect the presence of melibiose carrier in bacterial cells lysed on nitrocellulose filters. The amount of polyclonal antibody that was bound to the carboxyl-terminus of the melibiose transporter [8] was quantitated with [^{35}S]Protein A.

Table 1
Oligonucleotide primers used for site-directed mutagenesis

Oligonucleotide	Sequence
pC110S	5′-GGTCACGCtAACAAAGA-3′
pC235V	5′-CCCAAGAGgaCTGAAAGC-3′
pC310S	5′-AACACCACtGCTTAACA-3′
pC364S	5′-ACTTTCACtGCGTACGT-3′

2.6. Transport of sugars

Cells of DW1 containing a plasmid with either a normal or a mutant *melB* gene were grown overnight in LB medium that contained ampicillin at 100 μg ml^{-1}. In the morning, cells were diluted 50-fold and grown to mid-log phase at 37°C. Cells were harvested by centrifugation, washed twice with 100 mM MOPS buffer that was adjusted to pH 7.0 with Tris base and that contained 0.5 mM $MgSO_4$. The cells were resuspended in a volume of the same buffer to give a cell density corresponding to about 1 mg dry wt ml^{-1} (ca. 6×10^9 cells ml^{-1}). The transport reaction was started by the addition of radioactive sugar to the cell suspension that had been equilibrated to 22°C and, in experiments with sodium-coupled transport, equilibrated with 10 mM NaCl. At given time intervals, cell samples were filtered rapidly through 0.65 μm pore size nitrocellulose filters (Sartorius) and washed with 5 ml buffer. The filters were transferred to Liquiscint (National Diagnostics) and were counted in a liquid scintillation counter.

2.7. Sodium uptake

Sodium uptake was measured with a sodium-specific electrode (Radiometer Copenhagen G502Na) as described by Franco and Wilson [12]. The final cell density was 4–5 mg dry wt ml^{-1} (ca. 2×10^{10} cells ml^{-1}) and the final melibiose concentration was 5 mM.

2.8. Proton uptake

Uptake of protons was measured with a pH electrode (Radiometer GK2321-C) by the method of West [28]. Cell preparation in sodium free buffer was as described in Franco and Wilson [12] with the final cell density at 4–5 mg dry wt ml^{-1} (ca. 2×10^{10} cells ml^{-1}) and the final melibiose concentration at 3.3 mM.

2.9. Inhibition of transport by N-ethylmaleimide

The cell suspension (1–1.5 mg dry wt ml^{-1}) that was prepared as described for the transport of sugars was incubated with 0.5 mM *N*-ethylmaleimide for 10 min at 22°C. Dithiothreitol was then added to a final

concentration of 10 mM and the cells were tested for their ability to transport melibiose. For the mock treatment with *N*-ethylmaleimide, the dithiothreitol and *N*-ethylmaleimide were preincubated together for 10 min before the cells were added.

3. Results

3.1. Replacement of one or all cysteines in the melibiose carrier with serine or valine

The temperature resistant, wild type *melB* gene was used as the target of oligonucleotide site-directed mutagenesis as described in Materials and Methods. Four melibiose carriers of *E. coli* were constructed and each had a single cysteine changed to either serine (residue 110, 310 and 364) or valine (residue 235). Initially Cys235 was also changed to serine but this substitution resulted in a substantially reduced amount of transporter in the membrane: to 20% of the wild type amount. The replacement of all the cysteines by serine or valine was accomplished by 4 successive rounds of mutagenesis. The complete DNA sequence of all of the final plasmid constructs was determined to verify the presence of only the desired mutations.

3.2. Analysis of the relative amount of melibiose carrier produced by the melB genes with cysteine codon replacements

The relative amount of melibiose carrier present in the bacterial cell was analyzed with a polyclonal antibody directed against the carboxyl-terminus of the protein. The antibody reacting with a known quantity of lysed cells on a nitrocellulose filter was detected with radiolabeled Protein A as described in Section 2. As seen in Fig. 2, the alleles of *melB* that had mutations resulting in the replacement of either a single cysteine codon or all cysteine codons with a serine or valine codon produced an amount of carrier protein that was very similar to the amount produced by the original, wildtype gene.

3.3. Kinetic analysis of sodium-coupled sugar transport

The effect of the cysteine replacements in the melibiose carrier on the kinetics of sodium-coupled

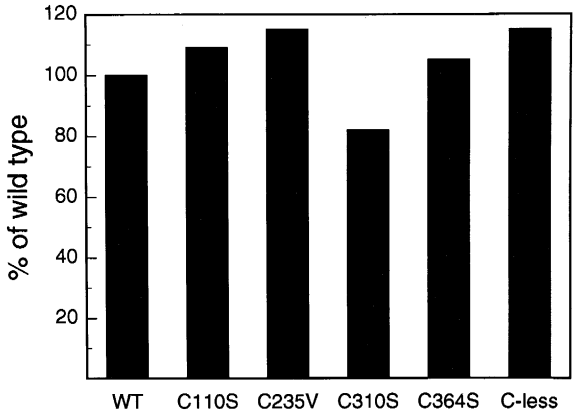

Fig. 2. Immunodetection of the relative amount of melibiose carrier. Melibiose carrier in bacteria harboring a plasmid with either a wild type or cysteine replacement *melB* gene was detected as described in Section 2 by using a polyclonal antibody directed against the carboxyl-terminus of the protein. The results are the average of 3–4 experiments with 4 data points per experiment.

accumulation of thiomethyl-β-galactoside or melibiose was examined. After trial experiments gave an estimated value for the K_m, seven different concentrations of sugar were selected that bracketed the estimate. The accumulation of sugar by the plasmid-harboring cells was then measured at each selected concentration after a 15 s incubation. The resulting data were analyzed by the double-reciprocal plot of Michaelis and Menten and the values for K_m and V_{max} were determined from the best fitting line that was obtained by linear regression analysis of the points. The results are presented in Table 2. The replacement of a single cysteine by serine or valine had little or no effect on the K_m of the melibiose carrier for either thiomethyl-β-galactoside or melibiose. The V_{max} of accumulation also was essentially unchanged except when thiomethyl-β-galactoside was the transported sugar for the two constructions that had the C364S replacement. In these two cases, the V_{max} was reduced to 24% of the wild type value when Cys364 was replaced with serine and was reduced to 49% of the wild type value in the cysteine-less melibiose carrier. However, when the V_{max} of sodium-coupled accumulation of melibiose was determined, the cysteine-less melibiose transporter shows only a modest (to 75% of the wild-type value) decrease compared to the wild type carrier.

Table 2
Kinetic constants for sodium-coupled transport

melB	TMG		Melibiose	
	K_m mM	V_{max} nmol min^{-1} mg^{-1}	K_m mM	V_{max} nmolmin^{-1} mg^{-1}
Wild type	0.07	76	0.3	120
C110S	0.08	77	0.3	110
C235V	0.07	97	0.2	100
C310S	0.06	71	0.2	85
C364S	0.1	18	0.2	120
Cys-less	0.1	37	0.3	89

Cells preparation and transport assays were as described in Section 2. Accumulation of sugar after 15 s was measured at 7 concentrations of radiolabeled thiomethyl-β-galactoside in the range of 20–400 μM or at 7 concentrations of radiolabeled melibiose in the range of 56–800 μM. The values for K_m and V_{max} are the average of at least 2 separate experiments with duplicate data points in each experiment.

3.4. Measurement of proton-coupled melibiose accumulation

The initial rate (at 15 s) and steady state (at 5 min) level of proton-coupled melibiose accumulation by the wild-type and the cysteine-replacement melibiose carriers is presented in Table 3. All of the cysteine-replacement strains tested can accumulate melibiose at least as well as the wild-type carrier. The melibiose carriers with a single cysteine replacement all show a slightly elevated initial rate of accumulation and, except for the C364S carrier, a slightly elevated steady state level of accumulation. The cysteine-less melibiose carrier has an initial rate of accumulation the same as wild-type whereas the steady state level of accumulation is ca. 1.9 times wild type.

Table 3
Initial rate and steady state proton-coupled accumulation of melibiose

melB	$[melibiose]_{in}/[melibiose]_{out}$	
	15 s	5 min
Wild type	1.6	4.7
C110S	2.3	5.7
C235V	2.3	6.2
C310S	2.1	4.8
C364S	2.0	7.2
Cys-less	1.6	9.0

Cell preparation and transport assays were as described in Section 2. The concentration of radiolabeled melibiose was 0.8 mM. The concentration of melibiose in the cell was calculated by assuming a cell volume of 0.4 μl for every 1×10^9 cells. The data are the results of at least 2 experiments with duplicate determinations in each experiment.

3.5. Measurement of melibiose-stimulated sodium uptake

The cotransport of sodium into the cell along with sugar can be measured with a sodium specific electrode as a decrease in external sodium concentration. The time course and magnitude of the change in

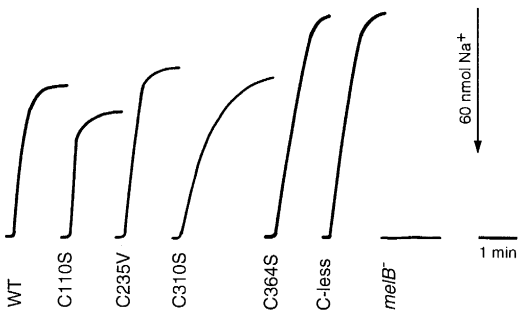

Fig. 3. Melibiose stimulated sodium uptake by bacteria harboring a plasmid with a wild type or mutant *melB* gene. Sodium movement was measured by a sodium electrode as described in Section 2 after addition of melibiose to a final concentration of 5 mM. Addition of 60 nmol of NaCl to the cell suspension resulted in an electrode response as indicated.

external sodium concentration upon melibiose uptake was thus compared among the various cysteine-replacement strains. Fig. 3 depicts the time course of the decrease in external sodium concentration of the wild-type and mutant carriers upon addition of melibiose (to a final concentration of 5 mM) to the cell suspension. The C110S, C235V, and C310S replacements in the melibiose carrier all resulted in a carrier that gave sodium movement similar to that of the wild-type carrier. The C364S substitution in the carrier resulted in a somewhat larger movement of sodium in response to melibiose compared to that seen in the wild-type carrier. Elevated movement of sodium was also seen with the cysteine-less melibiose carrier.

3.6. Measurement of melibiose-stimulated proton uptake

The cotransport of protons into the cell along with melibiose can be measured with a pH electrode as an alkalinization of the cell suspension. The time course and magnitude of the change in external pH was thus measured as described in Section 2. Fig. 4 depicts the time course and magnitude of the external pH change for the wild-type carrier and mutant carriers when melibiose was added to the cell suspension to a final concentration of 3.3 mM. The C110S, C310S, and C364S replacements in the melibiose carrier all resulted in carriers that gave a larger change in external pH in response to melibiose compared to the wild-type carrier. The C235V replacement gave a pH change that was similar to wild type and the cysteine-less carrier had somewhat reduced uptake of protons compared to wildtype.

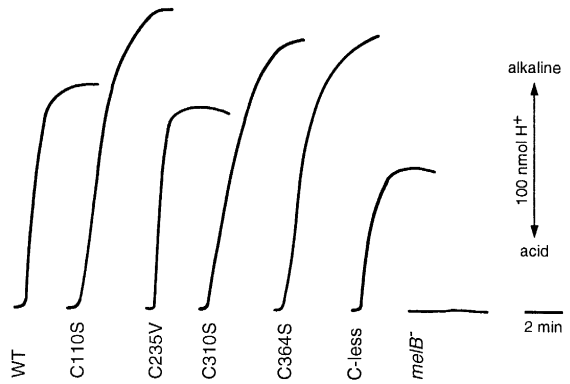

Fig. 4. Melibiose stimulated proton uptake by bacteria harboring a plasmid with a wild-type or mutant *melB* gene. Proton movement was measured by a pH electrode as described in Section 2 after addition of melibiose to a final concentration of 3.3 mM. Addition of 100 nmol of KOH to the cell suspension resulted in an electrode response as indicated.

Table 4
Inhibition by *N*-ethylmaleimide of sodium-coupled melibiose accumulation

melB	Reagent	Melibiose uptake nmol min^{-1} mg^{-1}	% Inhibition
Wild type	None	17.0	0
	NEM	2.6	85
C110S	None	21.1	0
	NEM	5.3	75
C235V	None	18.3	0
	NEM	3.0	84
C310S	None	17.3	0
	NEM	1.5	91
C364S	None	18.4	0
	NEM	15.4	16
Cys-less	None	15.1	0
	NEM	13.1	13

Cell preparation and transport assays were as described in Section 2. The concentration of radiolabeled melibiose was 0.1 mM and accumulation was measured after 1 min. Each data point in the table is the average of at least 3 experiments with triplicate determinations in each experiment.

3.7. The target of N-ethylmaleimide in the melibiose carrier

Previous studies of the melibiose carrier had demonstrated that sulfhydryl reagents completely inhibited the transport activity [21,29,30] and that substrates only partially protected against inhibition [21]. The construction of melibiose carriers with a single cysteine removed could potentially allow the identification of the cysteine responsible for the inhibitory action of the sulfhydryl reagents. The set of melibiose carriers that had a single cysteine replaced with another amino acid were thus treated with *N*-ethylmaleimide as described in Section 2 and the ability of

the treated carriers to accumulate melibiose in a sodium-coupled reaction was then measured. The results of these experiments are presented in Table 4. The replacement of Cys110, Cys235, or Cys310 resulted in a melibiose carrier that was still inhibited by *N*-ethylmaleimide. However, the replacement of Cys364 resulted in a carrier that now is largely unaffected by the sulfhydryl reagent. The small amount of inhibition of transport seen in the C364S and cysteine-less carrier could be due to reaction with amino or imide groups in the protein. It is unlikely due to inhibition of the respiratory chain because the addition of an alternate electron acceptor, reduced phenazine methosulfate [31], does not eliminate the inhibition (data not shown).

4. Discussion

We have constructed a set of 4 melibiose carriers each of which has one cysteine changed to either serine or valine. All of the mutant carriers exhibited transport activity that was similar to that of the wild type carrier. Elimination of Cys364 resulted in a melibiose carrier that was now unaffected by *N*-ethylmaleimide. The proposed topology of the melibiose carrier places Cys364 around the middle of a cytoplasmic loop that is 29 amino acids long and is between helices 10 and 11 [7]. The loop between helices 10 and 11 is also the location of mutants that alter substrate or cation specificity [13]. Thus, this cytoplasmic loop of the carrier appears to play a role in the transport process. In contrast, the cysteines that were identified as the targets of sulfhydryl reagents in the lactose permease [32,33], the glucose-6-P antiporter [17], and the EmrE antiporter [20] are found in regions of the protein that are assigned to transmembrane helices.

We have also constructed a melibiose carrier that has either serine or valine substituted for all 4 of the cysteines. The cysteine-less carrier exhibits appreciable sodium- or proton-coupled transport of sugars. In fact, the cysteine-less carrier has kinetic constants for the sodium-coupled accumulation of melibiose that are similar to those of the wild-type carrier. Cysteines are thus not essential for the transport of sugars by the melibiose carrier. The lack of a requirement for cysteinyl residues has been reported for the transport of substrates by other bacterial carriers: the lactose symporter [34], the glucose-6-P antiporter [17,18], and the multidrug antiporter, EmrE, from *E. coli*, [20]. The sodium-coupled transport of thiomethyl-β-galactoside, however, exhibited a reduction of the V_{max} to 49% of the wild type value. A reduction (to 24% of the wild type value) in the V_{max} for TMG transport was also seen with the single C364S substitution. In addition, the proton-coupled accumulation of melibiose at steady-state was about 2-fold greater with the cysteine-less carrier and the C364S carrier than with the wild-type carrier. One possible explanation for these results is that the recycling of the carrier is altered in the cysteine-less (and the C364S) carrier and thus alterations in the rate and level of accumulation are seen with no alteration in the binding of sugar. Further experiments to measure the component steps of the carrier cycle by measuring, for example, efflux or entrance counterflow in de-energized cells would test this hypothesis. Diminished efflux could also explain the elevated level of sodium movement seen in both the cysteine-less and the C364S carrier (Fig. 3). It is interesting to note that Pourcher et al. [35] have created mutants at Glu365 that show alterations in sugar efflux and exchange without alterations in binding of the sugar. In light of our current results and the past findings of others [13,35], further investigation of the importance of this cytoplasmic loop for the component steps of the carrier cycle would be most interesting.

The construction of a fully functional melibiose carrier that lacks cysteinyl residues now allows the introduction of cysteines at defined positions within the carrier. The susceptibility of these carriers to inhibition by membrane-permeable or membrane-impermeable sulfhydryl reagents and the reversal of the inhibition by substrates of the carrier allows the identification of amino acids surrounding the path that the transported sugar and accompanying cation take through the membrane. Additionally, as Wu and Kaback (lac carrier, [36]) and Lee et al. (trg chemoreceptor, [37]) have, for example, demonstrated, the introduction of two cysteines at defined locations and determination of their susceptibility to crosslinkage can address the question of what helices are adjacent to one another in the three-dimensional structure of the carrier in the membrane.

Acknowledgements

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