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The sodium-dependent D-glucose transport protein of *Helicobacter pylori*

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Abbreviations: dodecyl-β-D-maltoside, DDM.

Key words: glucose transport: membrane transport; *Helicobacter pylori*; sodium dependence

Summary

Helicobacter pylori is a Gram-negative pathogenic micro-aerophile with a particular tropism for the mucosal surface of the gastric epithelium. Despite its obligatory micro-aerophilic character, it can metabolise D-glucose and/or D-galactose in both oxidative and fermentative pathways via a Na^+-dependent secondary active transport, a glucokinase and enzymes of the pentose phosphate pathway. We have assigned the Na^+-dependent transport of glucose to the protein product of the *H. pylori 1174* gene. The gene was heterologously expressed in a glucose transport-deficient *Escherichia coli* strain, where transport activities of radiolabelled D-glucose, D-galactose and 2-deoxy-D-glucose were restored, consistent with the expected specificity of the hexose uptake system in *H. pylori*. D-Mannose was also identified as a substrate. The HP1174 transport protein was purified and reconstituted into proteoliposomes, where sodium-dependence of sugar transport activity was demonstrated. Additionally the tryptophan/tyrosine fluorescence of the purified protein showed quenching by 2-deoxy-D-glucose, D-mannose, D-glucose or D-galactose in the presence of sodium ions. This is the first reported purification and characterisation of an active glucose transport protein member of the TC 2.1.7 sub-group of the Major Facilitator Superfamily, constituting the route for entry of sugar nutrients into *H. pylori*. A model is derived of its 3D structure as a paradigm of the family.

Introduction

Helicobacter pylori is a common human pathogen, which predisposes individuals to gastric inflammation and a variety of diseases including gastric cancer (Goodwin *et al.*, 1986; Cover and Blaser, 1996; Blaser, 1997; Blaser and Berg, 2001). *H. pylori* can metabolise glucose via a glucokinase and enzymes of the pentose phosphate and glycolysis pathways (Mendz *et al.*, 1993; Chalk *et al.*, 1994; Hazell and Mendz, 1997). Glucose transport into intact cells of *H. pylori* was described by Mendz *et al.* (1995), who found that D-glucose and D-galactose were alternative physiological substrates, and that 2-deoxy-D-glucose was a useful non-physiological analogue for distinguishing activity from metabolism; also, sugar transport activity was stimulated by sodium ions (Mendz *et al.*, 1997).

Subsequently, sequencing of the genome of *H. pylori* 26995 confirmed the presence of the metabolic pathways for D-glucose, and revealed a portfolio of putative membrane transport proteins (Tomb *et al.*, 1997). Only one gene in the sequence, *hp1174* (Tomb *et al.*, 1997; Ren *et al.*, 1994), is likely to encode a glucose transport protein. Its predicted amino acid sequence has 45 % identity to the glucose/galactose transport protein, GluP, of *Brucella abortus* (Essenberg *et al.*, 1997), 22 % identity to the glucose/mannose transport protein of *Bacillus subtilis* (Paulsen *et al.*, 1998), and 32 % identity to the L-fucose-H$^+$ symport protein, FucP, of *Escherichia coli* (Bradley *et al.*, 1987; Zhe and Lin, 1989), for which there is a topological model based on experimental data (Gunn *et al.*, 1995).

Two strains of *H. pylori* have had their genome sequenced: 26695 and J99 (Alm *et al.*, 1999). They were compared by Alm *et al.* (1999), who found that the overall genomic organisation, gene order and predicted proteomes (sets of proteins encoded by the genome) of the two strains were similar (Alm *et al.*, 1999; Ge and Taylor, 1999). Only 6-7 % of the genes were specific to each strain, with almost half of these genes being clustered in a single hyper-variable region. The *hp1174* gene occurs in both with the same DNA sequence.

The isolation and characterisation of purified membrane transport proteins is technically challenging (Ward *et al.*, 2000; Saidijam *et al.*, 2003; Psakis *et al.*, 2007). Here we describe the amplified expression, purification and characterisation of the *H. pylori* HP1174 protein in *E. coli*. The isolated protein is active for transport

of D-glucose, provided that sodium ions are present, and this dependence was confirmed for glucose transport into the original *H. pylori* strain.

Results

Transport of glucose into H. pylori and its association with the hp1174 gene.

The transport of radiolabelled glucose into wild-type *H. pylori* was measured (Figure 1) and was reduced by about 5-fold when sodium ions were omitted from the medium (Figure 1).

The *hp1174* gene of *H. pylori* was inactivated by insertion of a kanamycin cassette (Experimental Procedures). This inactivation had the effect of reducing transport of glucose, even in the presence of sodium, to the low level observed in the wild-type without sodium (Figure 1), showing that the *hp1174* gene is encoding a protein that is responsible for the majority of transport of Na-glucose into *H. pylori*. Under rich medium conditions (Experimental Procedures), essential for growth of *H. pylori in vitro*, the doubling time of the mutant was 2.6 hours compared with 2.4 hours for the wild-type.

By varying the concentrations of the two substrates <u>and</u> using all the data points the K_m value for sodium (Figure S2, Supplementary information) was deduced to be 1.1 $\pm$ 0.4 mM and that for glucose was 0.8 $\pm$ 0.2 mM (Figure S3, Supplementary information). The V_{max} varied from 5.5 to 6.9 nmol.mg^{-1}.min^{-1} in two separate experiments. The least squares analyses shows some deviation from a fit to a single hyperbola for the glucose (Figure S3, Supplementary information), and there was a residual level of glucose transport in the mutant (Figure 1), perhaps reflecting the presence of a second much less active route for entry of glucose into the organism. Convergence to best-fit parameter values was not achieved when a fit to the sum of two hyperbolas was attempted, but qualitative estimates for the higher affinity system assigned to the HP1174 protein using a restricted set of measurements at the lower glucose concentrations yielded a K_m value in the range 0.026 - 0.162 $\pm$ 0.028 mM.

Characterisation of the glucose transport activity of the H. pylori hp1174 gene expressed in E. coli.

It is important to characterise the activity and specificity of the *H. pylori* protein expressed in *E. coli*, in order to ensure that its fundamental properties are not significantly affected by the addition of a small number of N- and C-terminal amino acids, especially the RGSH$_6$-tag, and by its new lipidic-environment (Tannaes and Bukholm, 2005; Psakis *et al*., 2007).

The activity of the *H. pylori* HP1174 protein expressed in *E. coli* was assessed by comparing the uptakes of radiolabelled glucose, mannose, galactose, and 2-deoxyglucose into IPTG-induced versus uninduced cells of the glucose/glactose transport-negative host strains transformed with the plasmid pTTQ18-*hp1174-RGSH$_6$* (Experimental Procedures).   In comparative experiments done on one batch of cells, transport of these sugars in the presence of 60 mM sodium was enhanced 5-10 fold by induction with IPTG (Figure 2A), confirming the preservation of both activity and specificity of the HP1174 protein expressed in *E. coli*.

When the cells were washed and resuspended in 150 mM KCl, 5 mM MES buffer the uptakes of glucose were reduced by up to 40%, and could be restored in the presence of 20-60 mM NaCl (data not shown).

The uptake of glucose was quite variable, increasing 2-10X in cells grown on IPTG, compared with uptakes into cells grown without IPTG. The extent of the uptake often tended to decline at higher IPTG concentrations and/or lengths of induction period. We attribute the latter phenomenon to some damaging effect of amplified expression of the HP1174- RGSH$_6$ protein on the membrane of the host cells, which also diminishes growth (data not shown). It is common, in our experience, for the energised uptake of substrates actually to be reduced in *E. coli* cells containing amplified levels of transport protein. Where the binding of substrate or inhibitor to the amplified protein can be checked, it is evident that the structure of the protein itself is not compromised. Rather, the energisation of transport, probably through enhanced leakiness of the membrane and reduced competence of energisation, is likely to be at fault.

The V_{max} for uptake of glucose into the host *E. coli* strain varied between 8.2 ± 0.6 to 29.5 ± 1.1 $\underline{nmol.mg^{-1}.min^{-1}}$, a little higher than that for *H. pylori* (above) for

<u>which</u> the apparent K_m was $0.0610 \pm 0.009 - 0.116 \pm 0.015$ mM (Figure S4, Supplementary information), perhaps reflecting a variable degree of effective energisation in different preparations

The uptake of D-glucose was inhibited by 2-deoxy-D-glucose, D-mannose, and D-galactose (Figure S5, Supplementary information), consistent with these sugars being alternative substrates for the *H. pylori* transport protein and with prior measurements of inhibition by sugars of glucose transport into the intact organism (Mendz *et al.*, 1995). Despite the similarity of the amino acid sequence of the *E. coli* L-fucose transport protein to the *H. pylori* HP1174-like proteins, inhibition of HP1174 activity by L-fucose was weak, and neither L-arabinose nor L-glucose inhibited transport by the cloned gene (Figure S5, Supplementary information), indicating that they are not substrates (c.f. Mendz *et al.*, 1995).

Despite extensive washing (Experimental Procedures), intact *E. coli* cells probably retained a significant level of endogenous Na^+, possibly due to the activity of endogenous Na^+/H^+ antiporters (Pinner *et al.*, 1993) and/or contamination (Kadner (1996); thus we were able to find only a modest enhancement (10-25 %) in glucose uptake by added Na^+ when measuring transport into those cells. The dependence of HP1174 activity upon Na^+ is examined again below using the purified protein.

It can be concluded that the specificity of the HP1174 glucose transport protein for sugars is essentially conserved when expressed in the *E. coli* host.

Amplified expression of the H. pylori 1174-RGSH$_6$ protein in E. coli

E. coli strain RE707 harbouring plasmid pTTQ18-*hp1174-RGSH$_6$* was grown on LB medium supplemented with 20 mM glycerol in the absence or presence of 0.2-1.0 mM IPTG. The IPTG consistently slowed the rate of growth (data not shown). A membrane preparation was made and the proteins separated by SDS-PAGE and stained with Coomassie blue. An extra protein band appeared in membranes from the IPTG-induced cells (Figure 2B). A Western blot of the same preparations showed a positive reaction in the corresponding molecular weight position only in the IPTG-induced host carrying the pTTQ18-*hp1174-RGSH$_6$* plasmid, and not in control preparations from the RE707 host cells (Figure 2C).

In general, glucose transport activity varied considerably from batch to batch of induced cells, ranging from 5 <u>to</u> 25 nmol.mg^{-1} at the 2 minute time point, while the level of HP1174 protein in the membrane rose to 14 % within one hour, and did not increase significantly thereafter (data not shown). So yield is increased only by prolonging growth to get more cells, rather than by further improvement of expression level.

The major inducible protein comprised about 12-14 % of the preparation containing mixed inner and outer membranes and migrated at an apparent Mr of 30-32 kDa (Figures 2B, 2C), corresponding to about 68 % of its predicted Mr. Most membrane transport proteins migrate anomalously in SDS-PAGE gels (Ward *et al.*, 2000), attributed to their partial unfolding in the presence of SDS. Evidence that the *hp1174-RGSH$_6$* gene product is intact is presented below.

Purification of the H. pylori 1174-RGSH$_6$ *protein.*

Inner membranes were prepared from 15-30 litre cultures of IPTG-induced *E. coli* RE707 containing plasmid pTTQ18-*hp1174-RGSH$_6$*. The proteins were solubilised in 1.0 % dodecyl-β-D-maltoside (Figure 3), and the HP1174-RGSH$_6$ protein was purified by nickel affinity chromatography (Experimental Procedures). In SDS-PAGE gels stained with silver or with Coomassie blue, the purified protein was >95% homogeneous as determined by densitometry (Figure 3A, B), albeit migrating at an anomalous molecular weight, as seen above, when using SDS-solubilised membranes. There was a lesser band of protein of higher Mr attributed to a completely unfolded state of the HP1174-RGSH$_6$ protein since it was also detected by Western blotting (Figure 3C). From one litre of cell culture, typically 2 ml of inner membrane were generated, containing 20 mg of protein, of which about 25 % (5 mg), was HP1174-RGSH$_6$. 1.6 mg of protein was recovered after purification.

The purified protein revealed a N-terminal amino acid sequence of *MNSH*MQKTSNTLALGSLTAL derived by automated Edman degradation, corresponding exactly to the sequence predicted from the construct, including the first four residues (italicized) derived from LacZ. The protein also reacted with the -RGSH$_6$ antibody, showing that the C-terminal region was present. Taken together,

these results show that the protein has retained all its amino acids in the purification process, and the question arose as to the integrity of its structure.

Reconstitution of transport activity and sodium dependence of the H. pylori glucose transport protein.

The protein was reconstituted into liposomes (Experimental Procedures), and its transport activity for glucose, D-mannose, D-galactose or 2-deoxy-D-glucose was measured (Figure 4A). The proteoliposomes, but not control liposomes, demonstrated a high level of glucose transport, followed by lower levels for mannose, 2-deoxy-D-glucose and D-galactose (Figure 4A - data for D-galactose is omitted for clarity).

Mendz *et al.* (1995) proposed that the transport of D-glucose into *H. pylori* was dependent upon the presence of Na^+ ions. Although an over 4-fold increase in glucose transport upon addition of sodium was demonstrated for the HP1174 protein in *H. pylori* (Figure 1), transport assays in *hp1174*-expressing *E. coli* cells revealed only a modest 40% increase in the presence of Na^+ (data not shown). We attributed the failure of observing a clear Na^+-dependence in intact *E. coli* cells to contamination by Na^+, even after extensive washing (Kadner, 1996). To address this apparent discrepancy, we reconstituted purified HP1174-RGSH$_6$ protein into liposomes and measured transport of sugars in the presence and absence of sodium. A clear dependence on Na^+ ions was seen for all four sugars (three shown in Figure 4). The activity in the absence of added sodium may be attributed to the difficulty of eliminating completely the presence of this cation, which may be present as a contaminant in the supporting media arising for example from the glass of containers (Kadner, 1996).

Secondary structure features of the purified HP1174-RGSH$_6$ protein.

The HP1174 protein contains 407 amino acids, which are predicted to be arranged as twelve transmembrane α-helices, with the N- and C-termini inside the cell consistent with its hydropathic profile (Figure S1, Supplementary information) and the experimentally determined model of the homologous *E. coli* FucP protein (Gunn *et al.*, 1995). The purified protein was therefore examined by circular dichroism (Greenfield and Fasman, 1969; Wallace *et al.*, 2003; Sreerama and Woody, 2004;), and by attenuated total reflection infra red spectroscopy

(Braiman and Rothschild, 1988; Vigano *et al.*, 2000, data not shown). Both these techniques yielded spectra (Figure 5 and data not shown) typical of a predominance of α-helical secondary structure as predicted from the protein's primary sequence (Figure S1, Supplementary information), which supports the genesis of an alpha-helical model of the protein (below). It also shows that the secondary structure of the protein had been preserved during the purification process.

Binding of sugars and sodium to the purified protein.

Transport proteins are likely to undergo profound conformational changes during their cycles of activity, and these may be revealed by changes in the fluorescence of tryptophan and tyrosine amino acid constituents, and/or fluorescent compounds that become attached to the protein (Walmsley *et al.*, 1994). When sugars were mixed with the isolated HP1174-RGSH$_6$ protein in the presence of Na$^+$ there was indeed a change in its emission spectra (Figure 6A) indicative of binding of the sugar to the protein. 2-Deoxy-D-glucose elicited a profound suppression of the protein's fluorescence (Figure 6A, B), with a K$_d$ of 0.8 mM and ΔF$_{max}$ of 56 %, indicative of conformational changes and/or interaction with the trpytophan residue as part of the substrate-binding cavity; the D-glucose, D-mannose, and D-galactose substrates were much less effective, K$_d$ 14.5 mM and ΔF$_{max}$ 38 % and K$_d$ 2.2 mM ΔF$_{max}$ 13 %, respectively (Figure 6A, B) (see Discussion).

Importantly, the dependence of the observed fluorescence changes on the binding of Na$^+$ was also investigated. Sodium on its own produced a significant quenching of fluorescence (Figure 6C), but this was markedly enhanced by the addition of the sugars (Figure 6C). Overall, there was a mutual enhancement of sodium binding in the presence of sugars, and of sugar binding in the presence of sodium, as expected of a random order of addition of sugar and cation substrates to the HP1174-RGSH$_6$ protein.

Analysis of a homology-based structural model of the HP1174 protein

A homology model (Figure 7) of the HP1174 protein was made using the crystal structure of *Escherichia coli* GlpT [PDB accession 1PW4; (Huang *et al.*, 2003)] as a template (Experimental Procedures). In addition to validation of the structure using MolProbity (Experimental Procedures), the surface of the chosen model was examined for the presence of hydrophilic side chains on the putative lipid-facing surfaces. These are shown in solid molecular representation (Figure 7B, D), with the truly polar residues shown in colour (red = acidic; blue = basic; green = amide). Overall, the model is reasonably distributed in this respect - there is a band of non-polar residues girdling the protein in the likely vicinity of the bilayer, with few polar residues to be found here.

Importantly, an aperture opening to the interior of the cell can be seen (Figure 7A, C), which is proposed to be the route for release of the glucose molecule into the cytoplasm after uptake from the exterior. Deep inside this cavity is located the single tryptophan found in the HP1174 protein (Figure 7E). The simplest interpretation of the fluorescence changes is that the hydrophobic face of the glucose interacts with the indole ring of this residue to quench its fluorescence. Alternatively, the position, and hence fluorescence, of the tryptophan may be altered by a conformational change caused by the binding of the glucose.

Discussion

The specificity of the cloned HP1174 for D-glucose, D-mannose, D-galactose and 2-deoxy-D-glucose and its dependence on sodium (found for *H. pylori* glucose transport, reconstitution assays for four substrates, and fluorescence measurements) confirmed that it is the protein responsible for the physiological uptake of sugars into *H. pylori* (Mendz *et al.*,1995; Marais *et al.*, 1999). The plasmid pTTQ18 (Stark, 1987), modified to incorporate fusion with the RGSH$_6$-tag (Ward *et al.*, 2000) has now proved useful for the amplified expression and purification of several such membrane proteins and a periplasmic protein from *H. pylori* (Saidijam *et al.*, 2003; Morrison *et al.*, 2003; Saidijam *et al.*, 2006; Shibayama *et al.*, 2007).

When expressed in *E. coli* the RGSH$_6$-tagged HP1174 protein appeared in the inner membrane of the cells, at an apparent molecular weight of 30-32 kDa. Expression of the *hp1174* gene was generally more reliable and occurred to a higher level in cells grown in LB medium, than in TY or minimal salts. The concentration of IPTG required for maximal induction of activity was in the range 0.2-0.5 mM, with a 2-3 hour period of induction. However, there was optimal expression of the protein after 1 hour.

Consistent with the location of HP1174 in the membranes, dodecyl-maltoside detergent was required for its solubilisation and purification. The purified protein could be reconstituted into artificial membranes, where it conferred sodium-dependent glucose transport activity. Despite its migration at an apparent molecular weight of 30-32 kDa when its predicted molecular weight is 45,543 [including the N-terminal addition of LacZ residues and C-terminal RGSH$_6$-tag], the protein was intact as shown by the correct amino acid sequence of the N-terminus plus a positive Western blot to its C-terminus. Such anomalous migration in SDS-PAGE is characteristic of membrane transport proteins Ward *et al.*, 2000; Saidijam *et al.*, 2006; Psakis *et al.*, 2007).

Both the CD and the IR spectra indicated an high α-helical content (55-85 % of the amino acid primary sequence), consistent with the topological model of the protein derived from its hydropathic profile (see below and Supplementary information) and alignment with an experimental model of the related L-fucose transport protein of *E. coli* (Gunn *et al.*, 1995). Importantly, these physical measurements, including its stability up to 45 $^{\circ}$C, demonstrate that the isolated protein is stable enough for crystallisation trials.

2-Deoxy-D-glucose elicited a substantial fluorescence change, indicative of a conformational change, in the HP1174 protein, while the D-glucose, D-mannose, or D-galactose substrates were less effective (Figure 6). These observations provide an extremely useful assay for monitoring the activity of the purified protein and possibly stabilising it during crystallisation trials. Further understanding of such conformational mobility and the molecular mechanism of the protein should come from elucidation of its three-dimensional structure through crystallisation and diffraction studies, which are being undertaken now the protein can be produced in sufficient quantities in a stable state. In the

meantime, insights can be provided by modelling the HP1174 protein on a homologous transporter of known structure. To this end, two members of the Major Facilitator Family from *E. coli* were considered as possible structural templates, the proton-lactose symporter LacY (Abramson *et al.*, 2003) and the glycerolP/Pi antiporter GlpT (Huang *et al.*, 2003). The latter was chosen for modelling in the present case because its glycerol phosphate substrate is more similar in size to the substrates of HP1174 than is lactose. The resultant model, made as described in Supplementary information, is shown in Figure 8. Such a model provides a rational basis, however imperfect, for the design of future experiments to elucidate the structure-activity relationships of the HP1174 protein, until such time as a 3-D structure can be realised from crystallographic or other methods. For example, the side-chain of the single tryptophan residue of HP1174, W269 in TM8, is predicted from the model to be exposed on the surface of the central hydrophilic cavity of the protein (Figure 7E). In this position it would be capable of direct interactions with permeants, consistent with the fluorescence change described above. Its possible functional importance is supported by the observation that the corresponding position is occupied by tryptophan in 28 of the 91 HP1174 homologues analysed in the present study, and by an aromatic residue (W, Y or F) in 49 of the sequences.

In addition to previous findings, where only glucose, and possibly galactose, appeared to be utilised by *H. pylori* (Mendz *et al.*, 1993; Marais *et al.*, 1999a), we showed for the first time that D-mannose can also be a substrate for the HP1174 transporter. The latter observation may be significant for infective competence, because in intestinal cells D-mannose, generated from D-glucose or provided in the diet, is used for the production of their mucosal glycoproteins, the role of which is to line and protect the epithelial cells (Smith *et al.*, 1990; Dromer *et al.*, 2002). If under energy-depletion conditions, *H. pylori* reverts to hexose uptake, it could compete with the host for the available D-mannose.

H. pylori is able to metabolise glucose by using the pentose phosphate pathway, glycolysis, and the primitive Enter-Doudoroff pathway as confirmed experimentally (Hazell and Mendz, 1997; Kelly, 2001) and also by analysing the whole genome sequence (Tomb *et al.*, 1997; Marais *et al.*, 1999b). Bioinformatics analysis of the *H. pylori* genome also indicated that HP1174 is probably the only transport protein for entry of sugars into the organism (Ren *et al.*, 2004); there is

no predicted phosphotransferase activity in *H. pylori* (Paulsen *et al.*, 1998), a common form of sugar transport in many bacteria (Martin and Russell, 1986; Mihara *et al.*, 2001). Nor is there predicted to be a sugar transport protein of the GLUT1 and SGLT families found in many bacteria and most organisms, including man (Baldwin and Henderson, 1989), which are the two major routes for entry of glucose and sugars into mammalian cells. The amino acid sequences of the GLUT and SGLT proteins do not show significant alignments with the HP1174 protein of *H. pylori*, and glucose transport into *H. pylori* is not susceptible to inhibitors that affect mammalian transporters (Mendz *et al.*, 1995). These observations raise the possibility of compromising the sugar metabolism of *H. pylori* infections by novel inhibitors of the HP1174 protein, which would not affect the sugar metabolism of host cells. The fluorescence changes we have discovered could be the basis of an high throughput assay to screen for such inhibitors.

Experimental Procedures

Materials- D-[2-^{3}H] glucose, D-[U-^{14}C] glucose, and D-[1-^{3}H] galactose were from Amersham, Buckinghamshire, HP7 9NA, UK. The *Escherichia coli* strains used throughout this study are detailed below (Maniatis *et al.*, 1982).

Plasmids

The plasmid used in this work is a derivative of the vector pTTQ18, which itself is a pUC derivative (Stark, 1987). It contains the hybrid *tac* promoter derived from the -35 region of p*trp* and the -10 region of p*lac,* a polylinker/*lacZα* region, the *bla* gene (encoding β-lactamase for the ampicillin resistance phenotype) and the *lacI^q* gene encoding the Lac repressor protein. It has also been modified to contain an oligonucleotide for introduction of an in-frame G_2RGSH_6 coding region at the 3' end of the gene to facilitate subsequent purification of the protein coupled with recognition of the protein by Western blotting (Ward *et al.*, 2000).

Growth media for bacteria

E. coli cells were cultured in Luria-Bertani (LB), 2 X tryptone yeast extract (2TY), or basal salts minimal medium (BM). Detailed compositions and preparation are given in Psakis (2005). All were supplemented with 20 mM glycerol, and with 100 $\mu g.ml^{-1}$ carbenicillin when hosting plasmids. A suspension of $A_{680} = 1.0$ was assumed to contain 0.68 mg dry mass per ml (Ashworth and Kornberg, 1966). *H. pylori* 26695 was grown in BHI medium supplemented with 10% bovine serum and collected when the A_{600} reached 0.789. A suspension of *H. pylori* cells of $A_{600} = 1.0$ was found to be of dry mass 0.289 mg.

Cloning

The PCR primers below were designed to amplify the *hp1174* gene from a sample of genomic DNA of *H. pylori* 26995 and introduce an *Eco*RI site (5'- AAG GAG AAT TCG CAT ATG CAA AAA ACT TCT AAC ACT CTG GCG CT –3') at the 5' end and a *Pst*I site (5'-GAA ACC CCC CCT CTG CAG CGG AGT TTT CTT CTT GC–3') at the 3' end.

The plasmid pTTQ18 containing the gene *norAHis$_6$* (Ward *et al.*, 2001) was digested with *Eco*RI and *Pst*I restriction endonucleases. The digested 4.59 kb fragment (pTTQ18 with the RGSH$_6$)-coding DNA sequence, observed size ~4.6 kb, and without the *norA* insert) was ligated to the *Eco*RI-*Pst*I digested j*hp1174* gene at various vector:insert molar ratios. A preliminary topological analysis indicated that the C-terminus of the HP1174 protein was likely to be located on the cytoplasmic side of the membrane (Supplementary data, Figure 1A), which is important because translocation of an H$_6$-tag across the membrane into the periplasm may be inimical to expression (unpublished observations). The ligation product (observed size, 5.7 kb) was transformed into *E. coli* strain XL1-blue, and recombinant clones were selected on LB plates containing 100 $\mu g.ml^{-1}$ carbenicillin. All four colonies tested by PCR screening were shown to contain the plasmid pTTQ18/*jhp1174-rgsh$_6$*

The purified plasmids were subjected to restriction enzyme analysis to confirm the identity and direction of the cloned gene, which was confirmed by automated DNA sequencing (D. Ashworth, University of Leeds). The plasmid construct was then transformed into *E. coli* strain RE707, which is impaired in the transport of D-

glucose, and strain RE777, which is impaired in the transport of D-galactose (Table 1; Essenberg *et al.*, 1997).

Induction by IPTG reduced the growth of the cells (Figure 2A). In a series of trials to find conditions optimal for production of HP1174-RGSH$_6$ protein, LB appeared to be superior to 2TY extract or BM medium, all with 20 mM glycerol added. Concentrations of IPTG from 0.2-1.0 mM were tried, which all had a similar inhibitory effect on growth (Figure 2A), and 0.4-0.8 mM seemed to be best for induction (data not shown). A period of two hours appeared to be optimal for appearance of activity without the risk of lysis of the cells, which tended to occur after 4 hours (not shown).

Membrane preparations

E. coli membrane preparations, from small scale culture volumes (50-500 ml), were made by the water lysis method (Ward *et al.*, 2000). Inner membrane preparations from 2-30 litre cultures were made by explosive decompression in a French Press followed by differential centrifugation using a sucrose gradient (Ward *et al.*, 2000).

Transport of radioisotope-labelled D-glucose into intact cells of H. pylori

H. pylori 26695 was grown in BHI medium supplemented with 10 % bovine serum and collected when the A$_{600}$ reached 0.789. The bacteria were washed with 5 mM MES pH 6.6 containing either 150 mM NaCl or KCl, and resuspended in 0.5 ml of the same buffer (0.5 ml per 1.5 ml of original culture). Uptake was initiated by adding 10 μl of D-[U^{14}C]-glucose (specific activity, 112 MBq.mmol^{-1}, 66 mM, GE Healthcare Biosciences) to the bacterial suspensions; final concentration was 1.32 mM (148 kBq.ml^{-1}). Samples were incubated at 37 ºC for 5 min. The bacterial cells were washed twice by centrifugation with the same buffer at 4 ºC, and resuspended in 0.1 ml of water. The experiments were done in triplicate. The bacterial suspensions were mixed with 10 ml of PCS Scintillation Cocktail (GE Healthcare Biosciences), and radioactivity was quantified by liquid scintillation counting.

Transport of radioisotope-labelled D-glucose into intact cells of E. coli

Cells grown in the indicated culture medium (LB/2TY), were harvested and washed three times with 5 mM MES pH 6.6 containing either 150 mM KCl (to assess Na$^+$ co-transport) or 60 mM NaCl, 90 mM KCl, before re-suspension in the same buffer to A$_{680}$ of 2.00 (Henderson *et al.*, 1977). Cells (0.5 ml) were energised by the addition of 5 µl 2 M glycerol and incubated with aeration at 25 ^{o}C. After 3 minutes, [1-^{3}H]-sugar or [1-^{14}C]-sugar was added to concentrations shown in the figures. At various time points 100-200 µl of solution were removed and applied to 0.45 µm cellulose nitrate filters (Whatman^{TM}). The filters were washed three times with the salts buffer above before the radioactivity was measured in the scintillation counter. All measurements were performed in duplicate.

Reconstitution of purified protein into preformed extruded liposomes

Liposomes (Ward *et al.*, 2000) were destabilized using 1.25 % (w/v) *n*-octyl-β-D-glucoside (OG), and then mixed with purified protein in 0.05 % n-dodecyl-β-D-maltoside (DDM) for 15 minutes at 4 ℃. The detergent was removed by the rapid dilution technique where the 2 ml of protein:lipid:detergent mixture was diluted to 130 ml with 50 mM KP$_i$ pH 7.6, 1 mM DTT, 20 mM sugar substrate, which takes the DDM below its critical micelle concentration (CMC). The proteoliposomes were recovered by centrifugation and resuspended in a maximum of 1 ml (total volume) 50 mM KPi, 1 mM DTT, 20 mM sugar, pH 7.6. For experiments with valinomycin the internal sugar was omitted.

Assays of sugar transport in proteoliposomes

Proteoliposomes (Ward *et al.*, 2000) were prepared as above. Protein (0.4 mg) solubilised in 0.05 % DDM was used per 4 mg of lipid [a lipid to protein ratio of 100:1 (w/w)]. For counterflow experiments at zero time, 40 µl of the sugar–loaded proteoliposomes were added to 920 µl of 50 mM KPi or NaPi buffer, 1 mM DTT, pH 7.6 plus 40 µl (100 µCi.ml^{-1}) of radiolabelled substrate to a final concentration of 0.802 mM. For K$^+$-valinomycin experiments sugar was omitted from the inside of the proteoliposomes, which contained 50 mM KPi, and were resuspended with or without valinomycin in 50 mM NaPi, 1 mM [^{3}H]-D-glucose and uptake of glucose measured. At each time point, an 80 µl sample was filtered on the vacuum

manifold using a 0.2 μm nitrocellulose filter (GSTF Millipore^{TM} filter). The filters were washed with an additional 2.5 ml of quench buffer and the radioactivity appearing in the proteoliposomes was determined by liquid scintillation counting.

Solubilisation of protein

 The membranes were diluted into 50 mM KP$_i$, pH 8.0, 2 mM β-mercaptoethanol and 10 % glycerol to yield a protein concentration of 5 mg.ml^{-1}. DDM (15% w/v stock solution) was then added drop-wise to give a final concentration of 1.0 % while stirring on ice for 30 min.

The solubilised membranes were centrifuged at 100,000 *g* for 1 hour, 4 °C. The supernatant containing solubilised membrane proteins was removed, and the pellet was washed with MQ water and resuspended with 400 μl of MQ water. A 100 μl sample of supernatant and the resuspended pellets were retained for analysis by SDS-PAGE (Laemli,1970).

Western blotting

Following SDS-PAGE electrophoresis, protein fractions were transferred to methanol activated PVDF membrane (Millipore^{TM}). For immunodetection of HP1174-RGSH$_6$, the RGSH$_4$-HRP conjugated mouse antibody (QIAGEN^{TM}) was used according to the manufacturer's instructions. Non-specific antibody binding to the membrane was blocked by addition of 5 % BSA in 1x TBST [washing buffer; 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 0.05 % Tween-20, 0.2 % Triton X-100]. Three washing steps (each lasting for 10 minutes) preceded and succeeded the incubation with the antibody. The membrane was finally incubated with BCIP/NBT solution (Chemicon^{TM}) for 2–5 min, prior to signal development.

Sequencing of amino acids in HP1174-RGSH$_6$

The identity of the HP1174-RGSH$_6$ protein was confirmed by automated Edman degradation of the N-terminal amino acids and ESI-MS/MS of partial peptides, performed by Dr. Jeff Keen (Protein Analysis facility, University of Leeds).

Purification of the $\underline{RGSH_6}$-tagged protein

The method is derived from that of Jung *et al.* (1998). The Ni-chelate was prepared for use by washing 1 ml (per 10 mg of protein) of the Ni-NTA resin three times in MQ water, then three times in wash buffer containing 50 mM KP_i pH 8.0, 300 mM NaCl, 2 mM ß-mercaptoethanol, 10 % glycerol, 10 mM imidazole pH 8.0, and a suitable detergent (Buffer A). The supernatant fraction from the solubilisation procedure was then incubated (with constant agitation) with the resin for 45 min to 1 hour at 4 °C. The resin was collected by centrifugation, <u>198 g</u>, 1min at room temperature and the supernatant retained for analysis by SDS-PAGE. The protein-resin complex was packed into a disposable column and the unbound protein was removed by washing the resin with 25 ml buffer E containing 10 mM imidazole pH 8.0. The resin was further washed with 20 ml buffer E containing 30 mM imidazole pH 8.0. The bound protein was eluted from the column with 5 ml buffer A containing 200 mM imidazole pH 8.0. The first 400 μl of eluted material was discarded and then four 1 ml fractions were collected. All washing steps were performed at 4 °C.

Circular Dichroism (CD) spectrum of purified $\underline{RGSH_6}$-tagged proteins

The purified protein was washed in 10mM phosphate pH 7.4, 0.05 % DDM, using a Centricon 100 (Amicon™), and resuspended to a final concentration of 30 $\mu g.ml^{-1}$. The samples were analysed in Hellma quartz glass cells of 1 mm path length in a Jasco model J-715 spectropolarimeter with constant flushing by nitrogen. Wavelengths were scanned from 190-260 nm at a rate of 50 $nm.min^{-1}$, and 20 spectra were accumulated and averaged. Similar spectra of buffer alone were subtracted from those of the protein solutions.

Thermal unfolding of the proteins was achieved by heating the samples between 10 °C and 90 °C at 10 °C intervals recording the effect of the temperature on the CD minima observed at 208 and 222 nm. Samples were then cooled down to 10 °C at 10 °C intervals, and the effects of temperature on the CD spectra were recorded again. Spectral changes were monitored for a 225-200 nm range (10 accumulations were collected per 10 °C interval). When the denatured protein sample was re-cooled down to 20 °C it's full spectrum was monitored as above for direct comparison to that of the native protein.

Spectrophotofluorimetry of purified H_6-tagged protein

Purified protein was diluted in buffer (50 mM NaPi/100 mM NaCl/1 mM EDTA, pH 7.4) to a final concentration of 150-250 µg.ml^{-1}. The conformational changes of purified HP1174-RGSH$_6$ upon binding of sugars were studied as an increase or decrease in fluorescence of tryptophan residues at 10 ^{o}C (Walmsley *et al.*, 1994), using a Jasco FP-6500 spectro-photo-fluorimeter. The suspension was allowed to mix for five minutes, prior to the addition of the ligand, and then for a further two minutes after the addition of the ligand. Excitation at 295 nm (2.5 nm slit width) and emission monitored at 300 and 400 nm (2.5 nm slit width) and scanning speed (250 nm.min^{-1}) were kept constant throughout the experiments to allow comparison of all data sets.

Generation of a homology model of the HP1174 protein

A homology model of the HP1174 protein was made using the crystal structure of *Escherichia coli* GlpT [PDB accession 1PW4; (Huang *et al.*, 2003)] as a template. Extensive sequence comparisons indicated that GlpT is a more likely paradigm than the structure of the Na$^+$-galactose transport protein from *Vibrio parahaemolyticus* (Faham *et al.*, 2008), the protein fold of which is very different from GlpT. Because of the evolutionary distance between HP1174 and the GlpT structural template, a combination of techniques was used to optimise their alignment. An initial alignment was created using the profile-to-profile based Multiple Mapping Method (Rai and Fiser, 2006). This preliminary alignment was next adjusted in the light of analysis of the aligned sequences of 91 HP1174 family members and, separately, of 100 GlpT homologues for patterns of residue conservation (using the ConSeq method, Berezin *et al.*, 2004), and of hydrophobicity. The resultant alignment is shown in Figure S6. Modeller version 8.2 (Fiser and Sali, 2003) was then used to create 100 models based on this alignment, and the five of lowest energy were further analysed using MolProbity (Lovell *et al.*, 2003). The one selected for subsequent investigation had only 6 residues in the disallowed region of the Ramachandran plot.

Acknowledgements

Figure legends

Fig. 1. Glucose transport by *Helicobacter pylori*. Transport of radioisotope-labelled glucose (0.8 mM) was measured by sedimentation as described in Methods: (circles), wild-type cells in 150 mM NaCl; (diamonds), wild-type cells in 150 mM KCl; or (triangles), HP1174 mutant cells in 150 mM NaCl.

Fig. 2. Activity, amplified expression and identification of the HP1174-$\underline{RGSH_6}$ protein in *E. coli*. *E. coli* RE707cells harboring the HP1174 plasmid were grown on LB/20 mM glycerol/50 µg.ml^{-1} carbenicillin-disodium and samples induced with IPTG (Methods) or uninduced were harvested, washed and resuspended in 60 mM NaCl, 90 mM KCL, 5 mM MES, pH 6.6. **A**. Transport of radioisotope-labelled sugar (0.1 mM) was measured into induced (closed symbols) or uninduced (open symbols) by filtration as described in Methods: (circles) glucose; (triangles) 2-deoxy-D-glucose; (squares) D-galactose [data for D-mannose has been omitted for clarity]. **B**. Mixed membranes were made from induced or uninduced cells (Methods) and protein samples (30µg) were examined by SDS-PAGE in a 15 % Coomassie blue stained gel: lane 1, molecular weight markers; lane 2, RE707 cells without plasmid; lane 3, uninduced cells with plasmid; and lane 4, cells with plasmid induced with IPTG. **C**. Western blot of the samples shown in **B**, using antibody that recognizes the RGSH$_6$ epitope at the protein's C-terminus.

Fig. 3. Purification of the *H. pylori* HP1174-$\underline{RGSH_6}$ protein. **A.** Samples of membrane fractions or solubilised material were loaded onto a 15 % SDS-PAGE gel. After electrophoresis to separate proteins, the gel was fixed and stained with silver. Lane 1, molecular weight standards; lane 2, predominantly outer membrane fraction; lane 3, predominantly inner membrane fraction, containing approximately 30% HP1174-$\underline{RGSH_6}$ as determined by densitometry; lane 4, non-solubilised fraction; lane 5, fraction solubilised in 1 % w/v dodecyl-β-D-maltoside, 300 mM NaCl, 10 mM Hepes, pH 7.9, 20 mM imidazole, 20 % glycerol; lane 6, unbound fractions collected after application of the soluble fraction to the NiNTA resin, washed out with 0.05 % w/v dodecyl-β-D-maltoside, 100 mM NaCl, 10 mM Hepes, pH 7.9, 20 mM imidazole, 10 % glycerol; lanes 7-11 HP1174-$\underline{RGSH_6}$ fractions

eluted with 0.05 % w/v dodecyl-β-D-maltoside, 10 mM Hepes, pH 7.9, 200 mM imidazole, 5 % glycerol. **B**. SDS-PAGE of purified protein stained with Coomassie Blue. **C**. SDS-PAGE of purified protein stained with antibody to the RGSH$_6$ epitope at the C-terminus of the purified HP1174 protein.

Fig. 4. Transport activity of the purified HP1174-RGSH$_6$ protein reconstituted into liposomes**.** The proteoliposomes and liposomes were prepared (Experimental Procedures) in 50 mM KPi, pH 7.6, 1 mM DTT, with 20.0 mM internal sugar. They were diluted into 50 mM KPi (open symbols) or 50 mM NaPi (closed symbols), pH 7.6, both containing 1 mM DTT and 0.8 mM external radioisotope-labelled sugar at the start of the reaction: (circles) glucose/glucose counterflow; (triangles) mannose/mannose counterflow; (squares) 2-deoxy-glucose/2-deoxy-glucose counterflow; (crosses) glucose/glucose counterflow using liposomes without protein in 50 mM - similar results for liposomes were obtained using the other sugars, but are not shown for clarity

Fig. 5. Secondary structure analysis of the purified HP1174-RGSH$_6$ protein – circular dichroism analysis. The purified protein was exchanged into 10 mM NaPi, pH 7.4, with 0.05 % DDM, and a solution of 30 μgml^{-1} was added to an Hellma quartz glass cell of 1mm path length. The circular dichroism spectrum was determined under constant nitrogen flushing at a scan rate of 50 nm.min^{-1} for from 190-260 nm for 20 accumulations in a Jasco model J-715 spectropolarimeter. The spectra from several independent preparations are consistent with an alpha-helical content of 70-85%.

Fig 6. Changes in fluorescence of purified HP1174-RGSH$_6$ from *H. pylori* when sugars and/or sodium bind. **A.** Purified HP1174-RGSH$_6$ (250 μg.ml^{-1}) in 50 mM NaPi/100 mM NaCl/1 mM EDTA, pH 7.4, 0.05 % DDM at 10 ^{o}C was excited at 295 nm and its emission spectra were recorded between 300-500 nm. The spectra shown, in decreasing fluorescence intensity, are: purified HP1174-RGSH$_6$ without added sugar; pure protein equilibrated with 47.62 mM D-galactose; pure protein equilibrated with 47.62 mM D-glucose; and pure protein equilibrated with 47.62 mM 2-deoxy-D-glucose. **B.** Binding curves for fluorescence changes for different

sugars added incrementally to the protein up to the concentrations shown. The parameters were calculated to be: K_d = 0.77 ± 0.05 mM and ΔFmax = 55.9 ± 0.6 for 2-deoxy-D-glucose binding (triangles); K_d = 14.5 ± 1.5 mM and ΔFmax = 37.5 ± 1.5 for D-glucose (circles); and K_d = 2.24 ± 0.32 mM and ΔFmax = 13.2 ± 0.4 for D-galactose (squares). **C**. Sodium chloride was added incrementally to HP1174-RGSH$_6$ protein up to the concentrations shown in 50 mM KPi, pH 7.6, 1 mM DTT, 0.05 % DDM plus 50 mM sugars as follows: (triangles) 2-deoxy-D-glucose; (circles) D-glucose; (diamonds) D-mannose; (squares) D-galactose; and (crosses) no sugar. The parameters for binding of NaCl were calculated to be: K_d = 0.121 ± 0.028 mM and ΔFmax = 12.37 ± 0.65 for NaCl with D-glucose; K_d = 0.046 ± 0.013 mM and ΔFmax = 17.25 ± 0.90 for NaCl with 2-deoxyD-glucose; K_d = 0.088 ± 0.024 mM and ΔFmax = 12.79 ± 0.76 for NaCl with D-mannose; K_d = 0.122 ± 0.027 mM and ΔFmax = 7.95 ± 0.0.39 for NaCl with D-galactose; and K_d = 1.48 ± 0.56 mM and ΔFmax = 9.65 ± 1.45 for NaCl without sugar.

Fig. 7. Three-dimensional model of the HP1174 protein from *H. pylori*. The proposed structure was derived from comparisons of aligned sequences of ~100 members of the TC 2.1.7 sub-group (Transport Classification Database) of the Major Facilitator Superfamily as described in Experimental Procedures and the Supplementary Information. The views in **A**, **B**, **C**, **D** are laterally from the membrane, with one side of the protein in the two top diagrams, and the opposite side in the lower two. **A, C**. The two left-hand diagrams represent the 12 helices as tubes coloured progressively: 1 and 7 yellow; 2 and 8 orange; 3 and 9 mauve; 4 and 10 pink; 5 and 11 green; and 6 and 12 blue. Such a scheme reflects the pseudo-symmetry of the two halves characteristic of the MFS transport proteins. **B, D**. The two right-hand diagrams are space-filling models, in which non-polar neutral residues are uncoloured, positively charged residues are blue, negatively charged residues are red, and neutral polar residues – glutamine and asparagines – are green. **E**. Model of HP1174 protein viewed from the extracellular face of the membrane showing the location of Trp269.

Table 1. Bacterial strains used in this study

Strain	Genotype	Source
RE707	Hfr (PO45) *thi Δ (pts-crr) galP gyrA Δ (recA-srlR) 306 srlR301::Tn10-84*	Essenberg *et al*., 1997
RE777	*his leu ilv Δlac mglP ΔgalP Δ(recA-srlR) 306 srlR301::Tn10-84*	Essenberg *et al*., 1997
BLR	*recA- ompT- (r$_B$ m$_B$) 306::Tn10 (Tc^r) [DE3 pLysS] (Cm^r)*	Novagen™
XL1 Blue	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [F'proAb lacI^q ZΔM15 Tn10 (Tet^R)]*	Stratagene™

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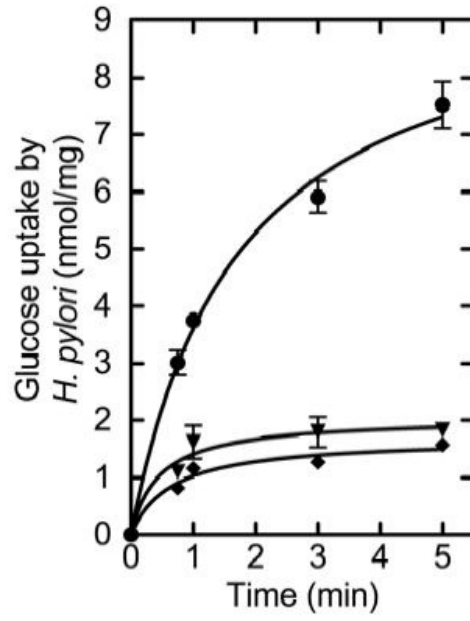
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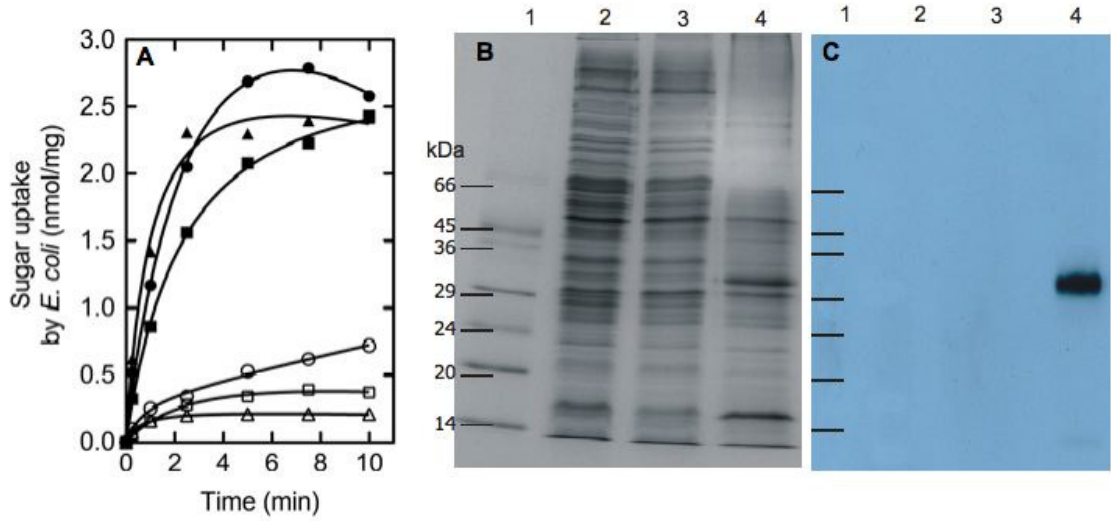
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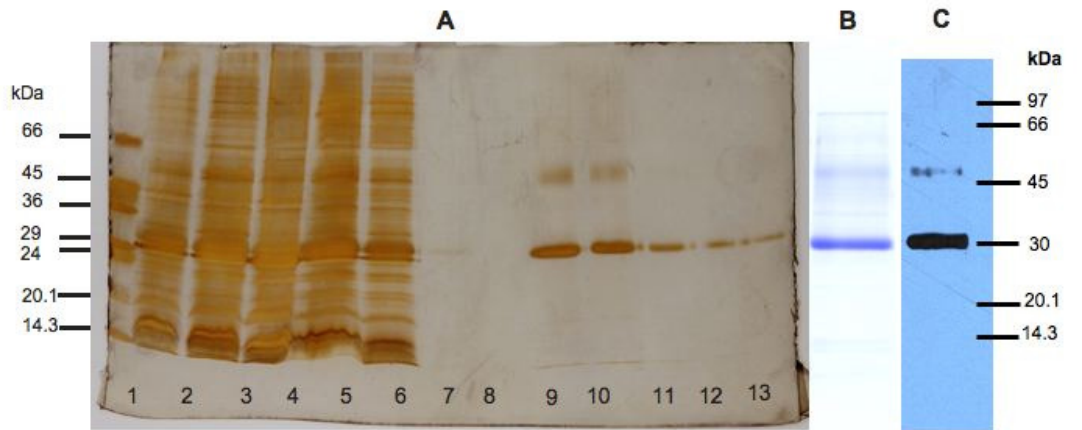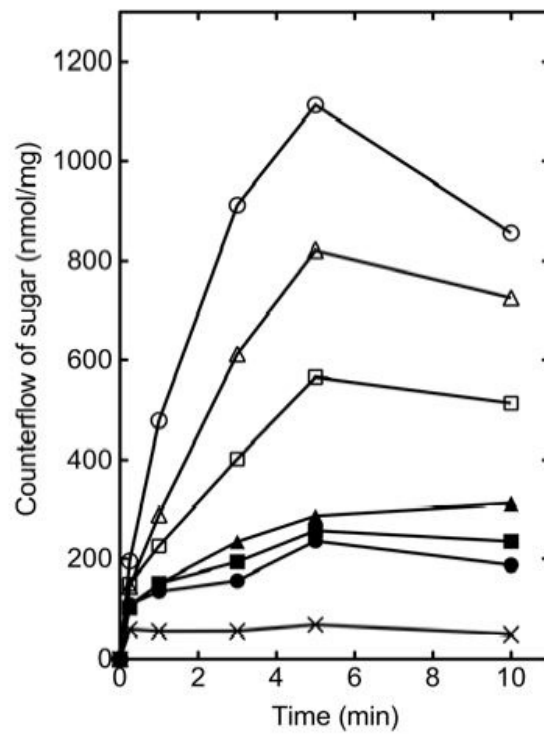
Henderson Figure 1
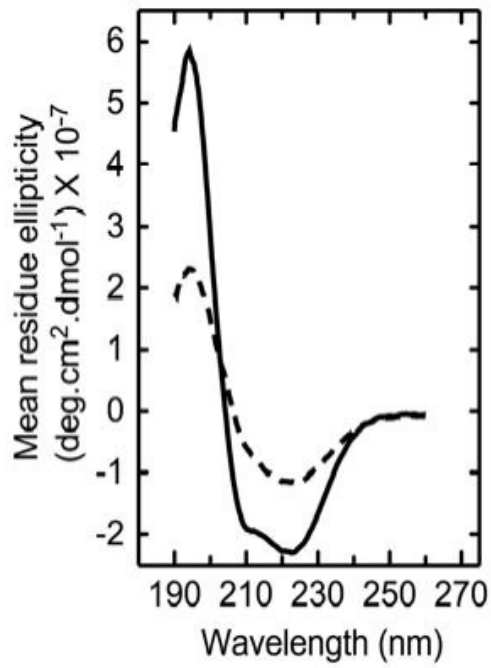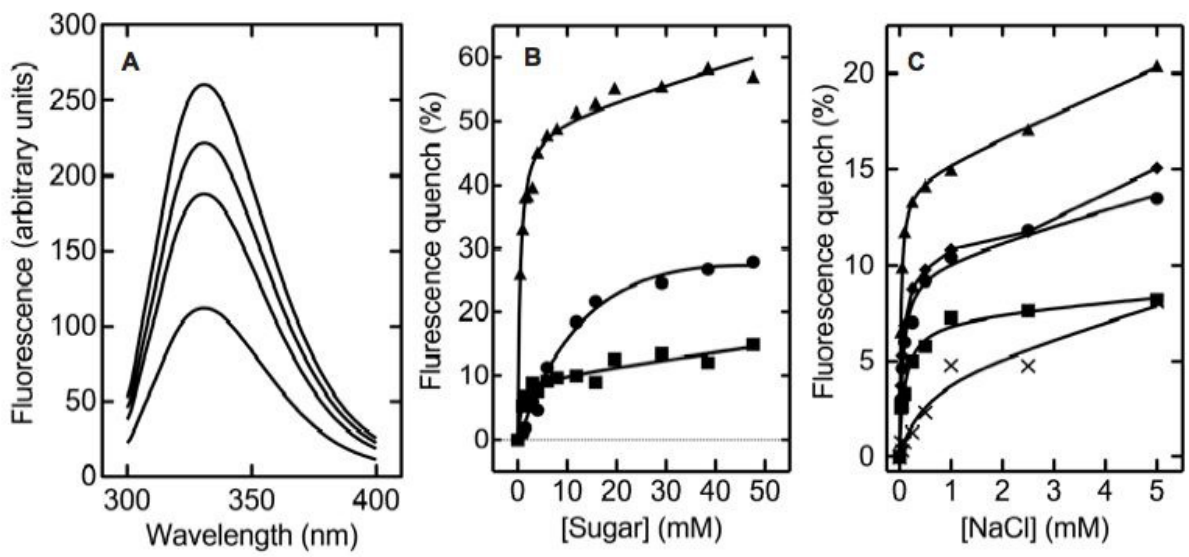

Henderson Figure 2

Henderson Figure 3

Henderson Figure 4