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

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Summary

Helicobacter pylori **is a Gram-negative pathogenic microaerophile with a particular tropism for the mucosal surface of the gastric epithelium. Despite its obligatory microaerophilic character, it can metabolize 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 characterization of an active glucose transport protein member of the TC 2.1.7 subgroup of the Major Facilitator Superfamily, constituting the route for entry of sugar nutrients into *H. pylori*. A model is derived of its three-dimensional 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; Goodwin, 1997; Blaser and Berg, 2001; Kavermann *et al*., 2003). *H. pylori* can metabolize glucose via a glucokinase and enzymes of the pentose phosphate and glycolysis pathways (Mendz *et al*., 1993; Chalk *et al*., 1994; Berg *et al*., 1997; 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 (Hazell and Mendz, 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*., 2004), 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; Clough, 2001), 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; Clough, 2001), for which there is a topological model based on experimental data (Gunn *et al*., 1995).

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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 organization, gene order and predicted proteomes (sets of proteins encoded by the genome) of the two strains were similar (Alm *et al*., 1999; Doig *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 characterization 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 characterization 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 (Fig. 1) and was reduced by about fivefold when sodium ions were omitted from the medium (Fig. 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 (Fig. 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 h compared with 2.4 h for the wild-type.

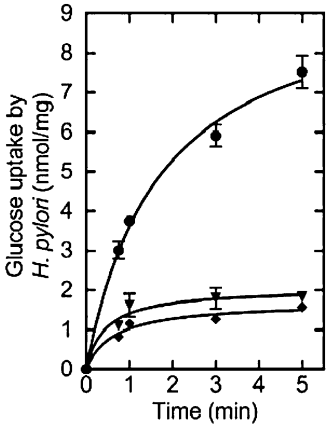

Fig. 1. Glucose transport by *Helicobacter pylori*. Transport of radioisotope-labelled glucose (0.8 mM) was measured as described in *Experimental procedures*: (circles) wild-type cells in 150 mM NaCl; (diamonds) wild-type cells in 150 mM KCl; or (triangles) HP1174 mutant cells in 150 mM NaCl.

By varying the concentrations of the two substrates and using all the data points the K_m value for sodium (Fig. S1, *Supporting information*) was deduced to be 1.1 ± 0.4 mM and that for glucose was 0.8 ± 0.2 mM (Fig. S2, *Supporting information*). The V_{max} varied from 5.5 to 6.9 nmol mg^{-1} min^{-1} in two separate experiments. The least squares analysis shows some deviation from a fit to a single hyperbola for the glucose (Fig. S2, *Supporting information*), and there was a residual level of glucose transport in the mutant (Fig. 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 ± 0.028 mM.

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

It is important to characterize 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 performed on one batch of cells, transport of these sugars in the presence of 60 mM sodium was enhanced 5- to 10-fold by induction with IPTG (Fig. 2A), confirming the preservation of both activity and specificity of the HP1174 protein expressed in *E. coli*.

When the cells were washed and re-suspended 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 2–10× in cells grown on IPTG, compared with uptakes into cells grown without IPTG. The extent of the uptake often

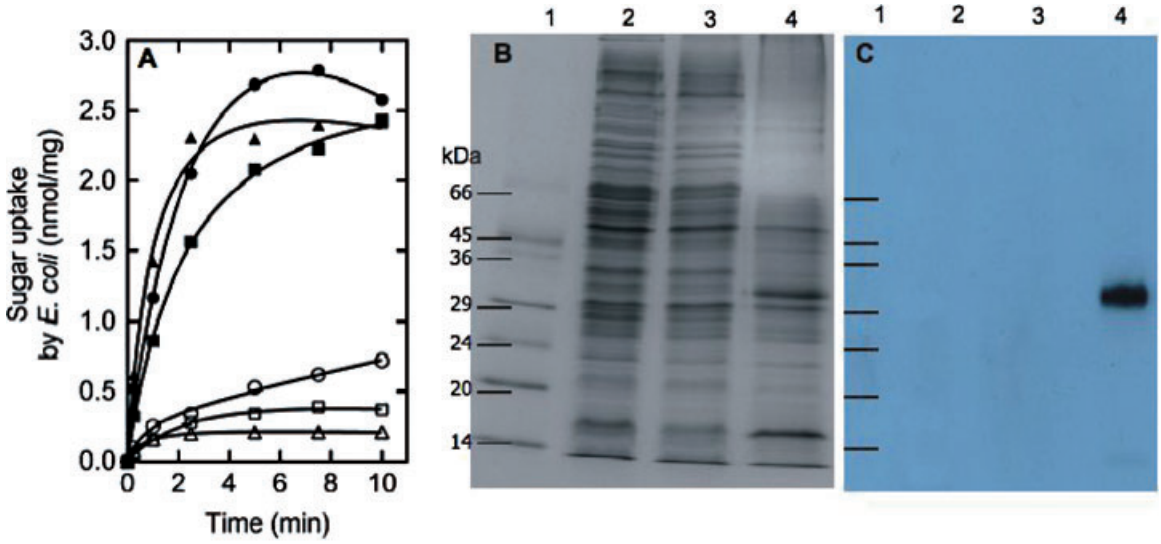

Fig. 2. Activity, amplified expression and identification of the HP1174-RGSH$_6$ protein in *E. coli*. *E. coli* RE707cells harbouring the HP1174 plasmid were grown on LB/20 mM glycerol/50 μg ml^{-1} carbenicillin-disodium and samples induced with IPTG (*Experimental procedures*) or uninduced were harvested, washed and re-suspended 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 *Experimental procedures*: (circles) glucose; (triangles) 2-deoxy-D-glucose; (squares) D-galactose (data for D-mannose have been omitted for clarity).
B. Mixed membranes were made from induced or uninduced cells (*Experimental procedures*) and protein samples (30 μg) 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.

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 energized 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 energization of transport, probably through enhanced leakiness of the membrane and reduced competence of energization, 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 and 29.5 ± 1.1 nmol mg^{-1} min^{-1}, a little higher than that for *H. pylori* (above) for which the apparent K_m was 0.0610 ± 0.009 to 0.116 ± 0.015 mM (Fig. S3, *Supporting information*), perhaps reflecting a variable degree of effective energization in different preparations

The uptake of D-glucose was inhibited by 2-deoxy-D-glucose, D-mannose and D-galactose (Fig. S4, *Supporting 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 (Fig. S4, *Supporting information*), indicating that they are not substrates (cf. 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; Griffith and Sansom, 1998) 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 on 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-*RGSH6 protein in* E. coli

Escherichia 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 (Fig. 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 (Fig. 2C).

In general, glucose transport activity varied considerably from batch to batch of induced cells, ranging from 5 to 25 nmol mg^{-1} at the 2 min time point, while the level of HP1174 protein in the membrane rose to 14% within 1 h, 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 molecular weight (Mr) of 30–32 kDa (Fig. 2B and C), 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 l of cultures of IPTG-induced *E. coli* RE707 containing plasmid pTTQ18-*hp1174-RGSH$_6$*. The proteins were solubilized in 1.0% dodecyl-β-D-maltoside (Fig. 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 (Fig. 3A and B), albeit migrating at an anomalous molecular weight, as seen above, when using SDS-solubilized 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 (Fig. 3C). From 1 l of cell culture, typically 2 ml of inner membrane was generated, containing 20 mg of protein, of which about 25% (5 mg) was HP1174-RGSH$_6$. Approximately 1.6 mg of protein was recovered after purification.

The purified protein revealed an 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

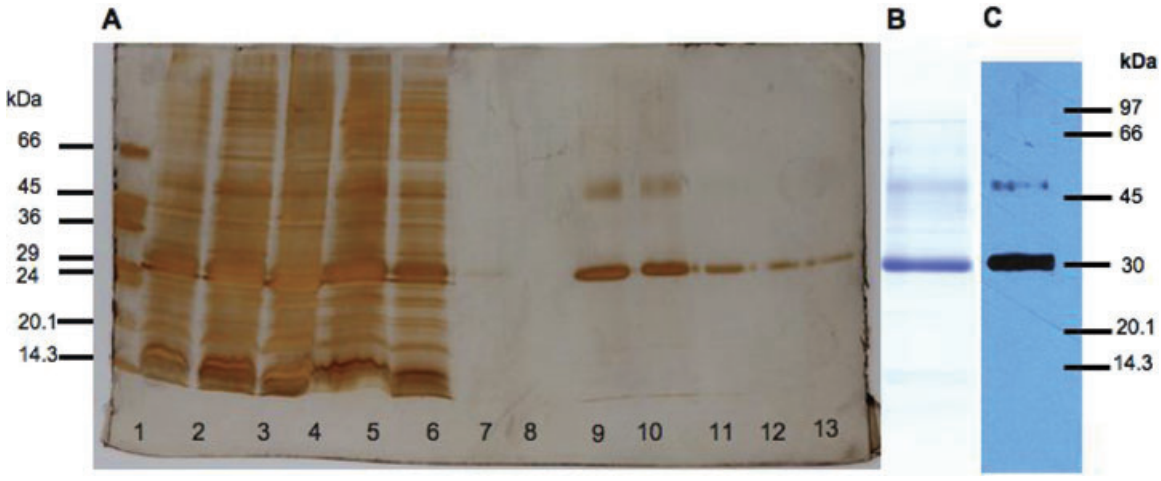

Fig. 3. Purification of the *H. pylori* HP1174-RGSH$_6$ protein.
A. Samples of membrane fractions or solubilized material were loaded onto a 15% SDS-PAGE gel. After electrophoresis to separate proteins, the gel was fixed and stained with silver. Lane 1, molecular weight standards; lane 2, predominantly outer membrane fraction; lane 3, predominantly inner membrane fraction, containing approximately 30% HP1174-RGSH$_6$ as determined by densitometry; lane 4, non-solubilized fraction; lane 5, fraction solubilized 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 7–13, HP1174-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.

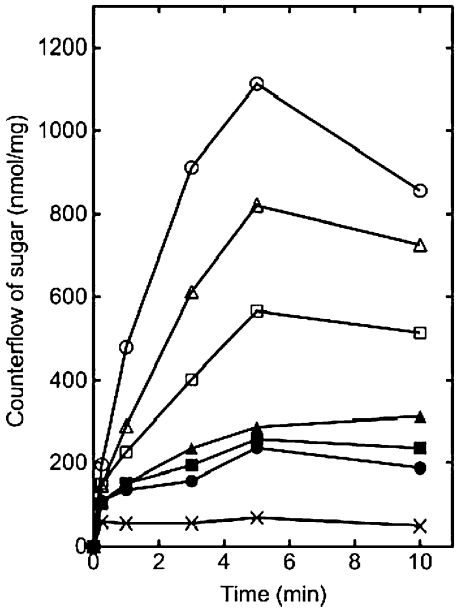

Fig. 4. Transport activity of the purified HP1174-RGSH$_6$ protein reconstituted into liposomes. The proteoliposomes and liposomes were prepared (*Experimental procedures*) in 50 mM KPi, pH 7.6, 1 mM DTT, with 20.0 mM internal sugar. They were diluted into 50 mM KPi (open symbols) or 50 mM NaPi (closed 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 – similar results for liposomes were obtained using the other sugars, but are not shown for clarity.

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 (Fig. 4). 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 (Fig. 4 – data for D-galactose are omitted for clarity).

Mendz *et al.* (1995) proposed that the transport of D-glucose into *H. pylori* was dependent on the presence of Na^+ ions. Although an over fourfold increase in glucose transport upon addition of sodium was demonstrated for the HP1174 protein in *H. pylori* (Fig. 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 Fig. 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 12 transmembrane α-helices, with the N- and C-termini inside the cell consistent with its hydropathic profile (Kyte and Doolittle, 1982; von Heijne, 1992; Hennig, 1999; Fig. S5, *Supporting 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 (CD) (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 (Fig. 5 and data not shown) typical of a predominance of α-helical secondary structure as predicted from the protein's primary sequence (Fig. S1, *Supporting information*), which supports the genesis of an

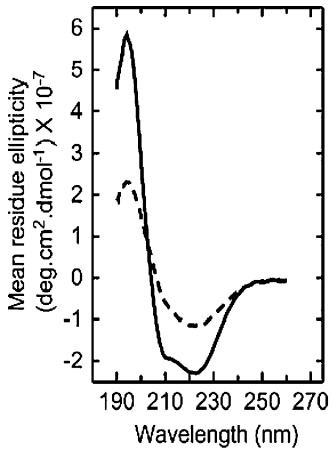

Fig. 5. Circular dichroism analysis of the purified HP1174-RGSH$_6$ protein in its native (solid line) and denatured (dashed line) states. The purified protein was exchanged into 10 mM NaPi, pH 7.4, with 0.05% DDM, and a solution of 30 μg ml^{-1} was added to a Hellma quartz glass cell of 1 mm path length.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

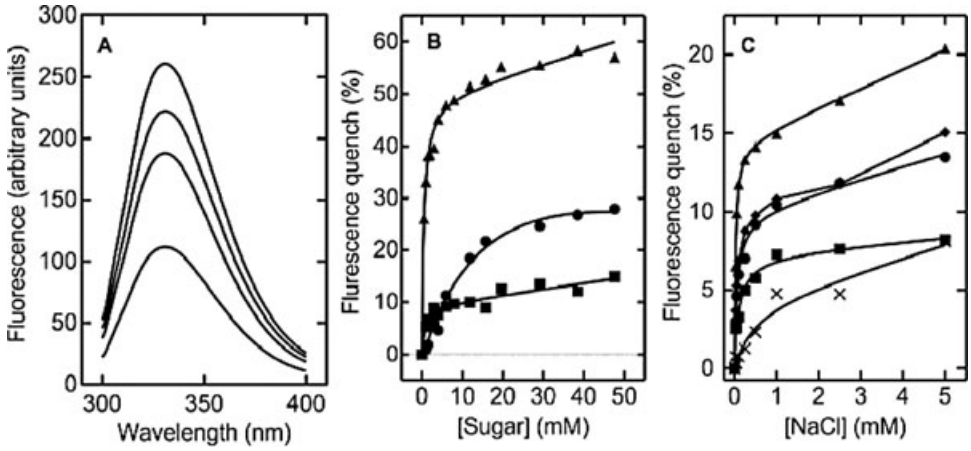

Fig. 6. Changes in fluorescence of purified HP1174-RGSH$_6$ from *H. pylori* when sugars and/or sodium bind.
A. Purified HP1174-RGSH$_6$ (250 μg ml^{-1}) in 50 mM NaPi/100 mM NaCl/1 mM EDTA, pH 7.4, 0.05% DDM at 10°C was excited at 295 nm and its emission spectra were recorded between 300 and 500 nm. 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: $K_d = 0.77 \pm 0.05$ mM and $\Delta F_{max} = 55.9 \pm 0.6$ for 2-deoxy-D-glucose binding (triangles); $K_d = 14.5 \pm 1.5$ mM and $\Delta F_{max} = 37.5 \pm 1.5$ for D-glucose (circles); and $K_d = 2.24 \pm 0.32$ mM and $\Delta F_{max} = 13.2 \pm 0.4$ 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: $K_d = 0.121 \pm 0.028$ mM and $\Delta F_{max} = 12.37 \pm 0.65$ for NaCl with D-glucose; $K_d = 0.046 \pm 0.013$ mM and $\Delta F_{max} = 17.25 \pm 0.90$ for NaCl with 2-deoxy-D-glucose; $K_d = 0.088 \pm 0.024$ mM and $\Delta F_{max} = 12.79 \pm 0.76$ for NaCl with D-mannose; $K_d = 0.122 \pm 0.027$ mM and $\Delta F_{max} = 7.95 \pm 0.0.39$ for NaCl with D-galactose; and $K_d = 1.48 \pm 0.56$ mM and $\Delta F_{max} = 9.65 \pm 1.45$ for NaCl without sugar.

α-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 (Fig. 6A) indicative of binding of the sugar to the protein. 2-Deoxy-D-glucose elicited a profound suppression of the protein's fluorescence (Fig. 6A and 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 (Fig. 6A and 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 (Fig. 6C), but this was markedly enhanced by the addition of the sugars (Fig. 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 (Fig. 7) of the HP1174 protein was made using the crystal structure of *E. 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 (Fig. 7B and 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 (Fig. 7A and 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

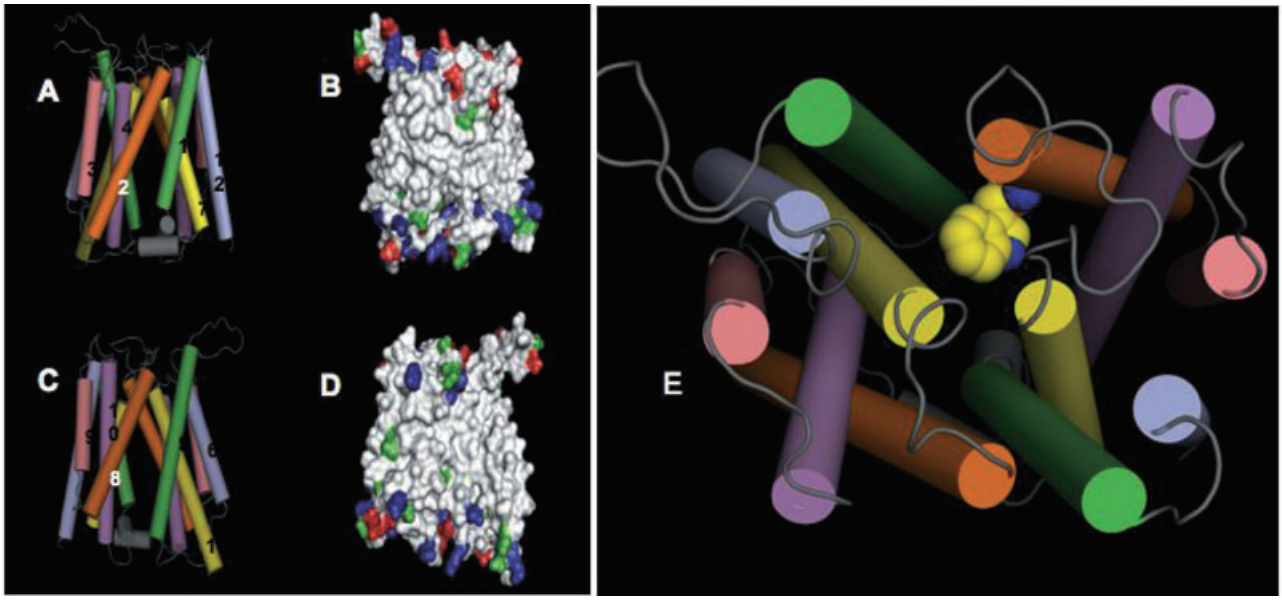

Fig. 7. Three-dimensional model of the HP1174 protein from *H. pylori*. The proposed structure was derived from comparisons of aligned sequences of ~100 members of the TC 2.1.7 subgroup (Transport Classification Database; Busch and Saier, 2002) of the Major Facilitator Superfamily as described in *Experimental procedures* and *Supporting information*. The views in (A)–(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 and 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 Major Facilitator Superfamily transport proteins.
B and 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.

is located the single tryptophan found in the HP1174 protein (Fig. 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 protein 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.*, 1999a). 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; 2006; Morrison *et al.*, 2003; 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 h period of induction. However, there was optimal expression of the protein after 1 h.

Consistent with the location of HP1174 in the membranes, dodecyl-maltoside detergent was required for its solubilization 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 infra-red spectra indicated a 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 *Supporting 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°C, demonstrate that the isolated protein is stable enough for crystallization 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 (Fig. 6). These observations provide an extremely useful assay for monitoring the activity of the purified protein and possibly stabilizing it during crystallization trials. Further understanding of such conformational mobility and the molecular mechanism of the protein should come from elucidation of its three-dimensional structure through crystallization and diffraction studies, which are being undertaken now as 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 Superfamily (Saier, 1998; Saier, 2000) 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 *Experimental procedures*, is shown in Fig. 7. Such a model provides a rational but imperfect basis for the design of future experiments to elucidate the structure–activity relationships of the HP1174 protein, until such time as a three-dimensional structure can be realized 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 (Fig. 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 utilized 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.

Helicobacter pylori is able to metabolize 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 (Pawlowski *et al*., 1999; 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 humans (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, UK. The *E. coli* strains used throughout this study are detailed below.

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/*lacZ*α 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).

Table 1. Bacterial strains used in this study

Strain	Genotype	Source
RE707	Hfr (PO45) *thi* Δ *(pts-crr) galP gyrA* Δ *(recA-srlR)* 306 *srlR*301::Tn*10-84*	Essenberg *et al.* (1997)
RE777	*his leu ilv* Δ*lac mglP* Δ*galP* Δ*(recA-srlR)* 306 *srlR*301::Tn*10-84*	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* Tn*10* (*Tet^R*)]	Stratagene™

Growth media for bacteria

Escherichia coli cells were cultured in Luria–Bertani (LB), 2× 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 μg 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 and its isogenic GluP mutant strain were grown in brain-heart infusion (BHI) supplemented with 10% fetal bovine serum under microaerophilic condition (5% O_2, 12% CO_2, 83% N_2) at 37°C with rotary shaking at 60 r.p.m. The bacterial growth was monitored by measuring absorbance (A) at a wavelength of 600 nm. An overrnight liquid culture of A_{600} 1.9, was used as inoculum to start the culture was in two 15 cm dishes containing 40 ml of fresh medium. In one dish 1 ml of inoculum culture was added and in the other dish 2 ml of inoculum culture was added. The bacterial growth was monitored up to 26.5 h. Doubling time was estimated from the linear range of the growth line on log scale plot, where the growth was actually exponential.

Construction of an HP1174-deficient mutant of H. pylori *26695*

A 749 bp *Bam*HI-*Hin*d III fragment containing the HP1174 gene from nucleotide 289 to 1037 was cloned into plasmid pUC18. A kanamycin-resistance gene cassette was inserted into the cloned gene using the EZ-Tn5 <KAN-2> Insertion Kit (EPICENTRE, Wisconsin, U.S.A., EZI982K). The plasmid was then transformed into *H. pylori* 26695 by electroporation. The transformants were incubated for 4 days on a BHI agar plate with 10% bovine serum and 25 mg ml^{-1} kanamycin. Several clones, in which the HP1174 gene of the chromosome was disrupted by homologous recombination, were obtained. The insertion of the kanamycin resistance gene within the HP1174 gene was confirmed by PCR and subsequent nucleotide sequencing. The cassette was found to be inserted next to G849 of the HP1174 gene.

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 EcoRI site (5′-AAG GAG AAT TCG CAT ATG CAA AAA ACT TCT AAC ACT CTG GCG CT-3′) at the 5′ end and a PstI 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 EcoRI and PstI 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 EcoRI–PstI-digested *jhp1174* 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 (*Supporting information*, Fig. 1A), which is important because translocation of an H$_6$-tag across the membrane into the periplasm may be inimical to expression (P.J.F. Henderson; A. Ward; G. Psakis, unpubl. obs.). 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 μg 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. 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 to 1.0 mM were tried, which all had a similar inhibitory effect on growth, and 0.4–0.8 mM seemed to be the best for induction (data not shown). A period of 2 h appeared to be optimal for appearance of activity without the risk of lysis of the cells, which tended to occur after 4 h (not shown).

Membrane preparations

Escherichia 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 l of 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 wild-type and HP1174-deficient H. pylori

H. pylori 26695 and its HP1174-deficient isogenic mutant were grown in BHI medium supplemented with 10% bovine serum and collected when the A_{680} reached 0.9. The bacterial cells

were washed with 5 mM MES pH 6.6 containing either NaCl or KCl at the indicated concentrations, and resuspended in 1.5 ml of the same buffer, the A_{680} of which was adjusted to 2.0. Uptake was initiated by adding 20 µl of D-[U^{14}C]-glucose (specific activity, 111 MBq mmol^{-1}, 66.7 mM, GE Healthcare Biosciences, Buckinghamshire, UK, code number CFB2, Batch 263) to 1.5 ml of the bacterial suspensions; final concentration was 0.88 mM (98.7 kBqml^{-1}). Samples were incubated at 37°C. At 45 sec, 1 min, 3 min, and 5 min, 0.1 ml of the suspension was transferred to a 1.5 ml tube containing 1 ml of silicone oil and bacterial cells were separated immediately from the substrate-containing buffer by centrifugation. Then the samples were kept on dry ice, by which the upper buffer phase and the bacterial pellet at the bottom of the tube were kept frozen. The frozen buffer and silicone oil were discarded, and the bacterial pellet was resuspended in 50 ml of water. The bacterial suspensions were mixed with 5 ml of PCS Scintillation Cocktail (GE Healthcare Biosciences, code number NPCS104-1CS), and radioactivity was quantified by liquid scintillation counting. The experiments were done in triplicate.

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; Henderson and MacPherson, 1986). Cells (0.5 ml) were energized by the addition of 5 µl of 2 M glycerol and incubated with aeration at 25°C. After 3 min, [1-^{3}H]-sugar or [1-^{14}C]-sugar was added to concentrations shown (Fig. 2A, Figs S3 and S4). At various time points 100–200 µl of solution was removed and applied to 0.45 µm cellulose nitrate filters (Whatman™). 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 pre-formed extruded liposomes

Liposomes (Mayer *et al*., 1986; Ward *et al*., 2000; Rigaud and Levy, 2003) 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 min at 4°C. 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 KPi 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 re-suspended in a maximum of 1 ml (total volume) of 50 mM KPi, 1 mM DTT, 20 mM sugar, pH 7.6.

Assays of sugar transport in proteoliposomes

Proteoliposomes (Ward *et al*., 2000) were prepared as above. Protein (0.4 mg) solubilized 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. At each time point, an 80 µl sample was filtered on the vacuum manifold using a 0.2 µm nitrocellulose filter (GSTF Millipore™ 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.

Solubilization of protein

The membranes were diluted into 50 mM KPi, 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 solubilized membranes were centrifuged at 100 000 *g* for 1 h, 4°C. The supernatant containing solubilized membrane proteins was removed, and the pellet was washed with MQ water and re-suspended with 400 µl of MQ water. A 100 µl sample of supernatant and the re-suspended 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™). For immunodetection of HP1174-RGSH$_6$, the RGSH$_4$-HRP-conjugated mouse antibody (QIAGEN™) was used according to the manufacturer's instructions. Non-specific antibody binding to the membrane was blocked by addition of 5% BSA in 1× 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 min) preceded and succeeded the incubation with the antibody. The membrane was finally incubated with BCIP/NBT solution (Chemicon™) 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 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 KPi pH 8.0, 300 mM NaCl, 2 mM β-mercaptoethanol, 10% glycerol, 10 mM imidazole pH 8.0 and a suitable detergent (buffer A). The supernatant fraction from the solubilization procedure was then incubated (with constant agitation) with the resin for 45 min to 1 h at 4°C. The resin was collected by centrifuga-

tion, 198 *g*, 1 min 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 of buffer A containing 10 mM imidazole pH 8.0. The resin was further washed with 20 ml of buffer A containing 30 mM imidazole pH 8.0. The bound protein was eluted from the column with 5 ml of 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.

CD spectrum of purified $RGSH_6$-tagged proteins

The purified protein was washed in 10 mM phosphate pH 7.4, 0.05% DDM, using a Centricon 100 (Amicon™), and re-suspended to a final concentration of 30 μg 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 to 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 its 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 a decrease in fluorescence of tryptophan residues at 10°C (Walmsley *et al*., 1994), using a Jasco FP-6500 spectrophotofluorimeter. The suspension was allowed to mix for 5 min, prior to the addition of the ligand, and then for a further 2 min 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 *E. 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 optimize 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 Fig. 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 six residues in the disallowed region of the Ramachandran plot.

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