Cysteine residues in the D-galactose–H^+ symport protein of *Escherichia coli*: effects of mutagenesis on transport, reaction with *N*-ethylmaleimide and antibiotic binding

Terence P. McDONALD[1] and Peter J. F. HENDERSON[2]
School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K.

The galactose–H^+ membrane-transport protein, GalP, of *Escherichia coli* is similar in substrate specificity and susceptibility to cytochalasin B and forskolin, to the human GLUT1 sugar-transport protein; furthermore, they are about 30% identical in amino acid sequence. Transport activities of both GalP and GLUT1 are inhibited by the thiol-group-specific reagent, *N*-ethylmaleimide. GalP contains only three cysteine residues at positions 19, 374 and 389, each of which we have mutated, singly and in combination, to serine. Each single change of Cys → Ser has only a minor effect on transport activity, whereas alteration of all three simultaneously profoundly diminishes V_{max} for transport. The high level of expression of the GalP protein facilitates measurements of the reactivity of each mutant with *N*-ethylmaleimide or eosin 5-maleimide, which conclusively demonstrate that Cys^{374} is the site of covalent modification by the reagents. By comparing the reactivity of Cys^{374} in right-side-out and inside-out vesicles it appears that Cys^{374} is located on the cytoplasmic face of the GalP protein. Although impaired in transport activity, the 'Cys-free' mutant, with all three cysteine residues mutated into serine, binds cytochalasin B and forskolin with wild-type affinities. All these results are interpreted in terms of a 12-helix model of the folding of the protein, in which the relative orientations of helix 10, containing the reactive Cys^{374} residue, and helix 11, containing the unreactive Cys^{389} residue, can now be defined.

Key words: glucose transporter, membrane transport, sugar–H^+ symporter, thiol-group-specific reagent.

INTRODUCTION

The D-galactose–H^+ symport protein (GalP) from *Escherichia coli* is homologous to the L-arabinose-H^+ symporter (AraE) and the D-xylose-H^+ symporters (XylE) of *E. coli*, with 64% and 33% identity respectively [1,2]. These secondary active transporters are also homologous to a family of passive facilitative transporters for glucose (GLUT) in mammals, and to other sugar-transport proteins in diverse organisms [2–9]. The inclusion of both active and passive transporters in the homologous transporter family implies they share common features of both structure and molecular mechanism. Furthermore, the *E. coli* and mammalian proteins are predicted to have a similar membrane topology, comprising 12 membrane spanning α-helices (12 TM), with helices 6 and 7 connected by a cytoplasmic domain containing 60–70 amino acids (Figure 1; [2,8,10]). The sugar specificities of the *E. coli* transporters differ, with GalP primarily transporting hexoses, but with the AraE and XylE transporting primarily pentoses. However, the sugar specificity of GalP is very similar to that of the glucose transporters from human erythrocytes (GLUT1) and rat adipocytes (GLUT4) [11–14], giving rise to the suggestion that GalP is the bacterial equivalent of the mammalian glucose transporter [9]. This suggestion is reinforced by the observation that sugar transport mediated by either GLUT1 or GalP is inhibited by the antibiotics cytochalasin B and forskolin [13–21]. Sugar transport via AraE is inhibited by cytochalasin B [22], but not forskolin [18], whereas neither antibiotic inhibits XylE (T. P. McDonald, G. E. M. Martin and P. J. F. Henderson, unpublished work). Since sugars displace cytochalasin B and forskolin, such antibiotic specificities may reflect differences in the sugar-binding sites of these transporters.

When incubated with the thiol-directed reagent *N*-ethylmaleimide (NEM), for 15 min at 25 °C, GalP, XylE and AraE lose 70–90% of their activity in a substrate-protected manner [23–26]. GLUT1 is also inhibited by NEM [27]. However, in this case the presence of D-glucose accelerates the rate of inactivation [27,28]. The implication of all these results is that the environment of one or more thiol groups is/are affected by the binding of sugar and that the residue(s) may be at or near the sugar-binding site.

The close homology between GalP and AraE is reflected in the conservation of Cys residues. GalP is predicted from the gene sequence to contain three Cys residues [2], whereas AraE is predicted to have five [1]. Two of the Cys residues in GalP are conserved in AraE {Cys^{374}[transmembrane helix (TM) 10] and Cys^{389} (TM11)}. XylE is predicted to have seven Cys residues [29] and GLUT1 six [30]. The two Cys residues conserved between GalP and AraE are the only ones that are conserved between the four homologous proteins. In order to investigate the role of Cys residues in the reaction with NEM, transport and antibiotic binding, the cysteine residues in GalP were changed to serine residues by oligonucleotide mutagenesis.

MATERIALS AND METHODS

Bacterial strains

E. coli strain JM1100 *HfrC his-gnd*$^{\Delta}$ *thyA galK ptsM galP mglP ptsF ptsG* [31], containing plasmids with wild-type or mutated

Abbreviations used: GalP, the galactose-H^+ membrane transport protein of *Escherichia coli*; GLUT1, a mammalian glucose transport protein; AraE, L-arabinose–H^+ symporter; XylE, D-xylose–H^+ symporter; NEM, *N*-ethylmaleimide; ISO, inside-out; RSO, right-side-out; TM, transmembrane helix; throughout this paper numbering of amino acid residues refers to their positions in the sequence of GalP; K_m and V_{max} values are apparent, as it was not known whether the co-substrate, H^+, was at saturating amounts.

[1] Present address: Institute of Virology, University of Glasgow, Church Street, Glasgow GTT 5JR, Scotland, U.K.
[2] To whom correspondence should be addressed (e-mail p.j.f.henderson@leeds.ac.uk).

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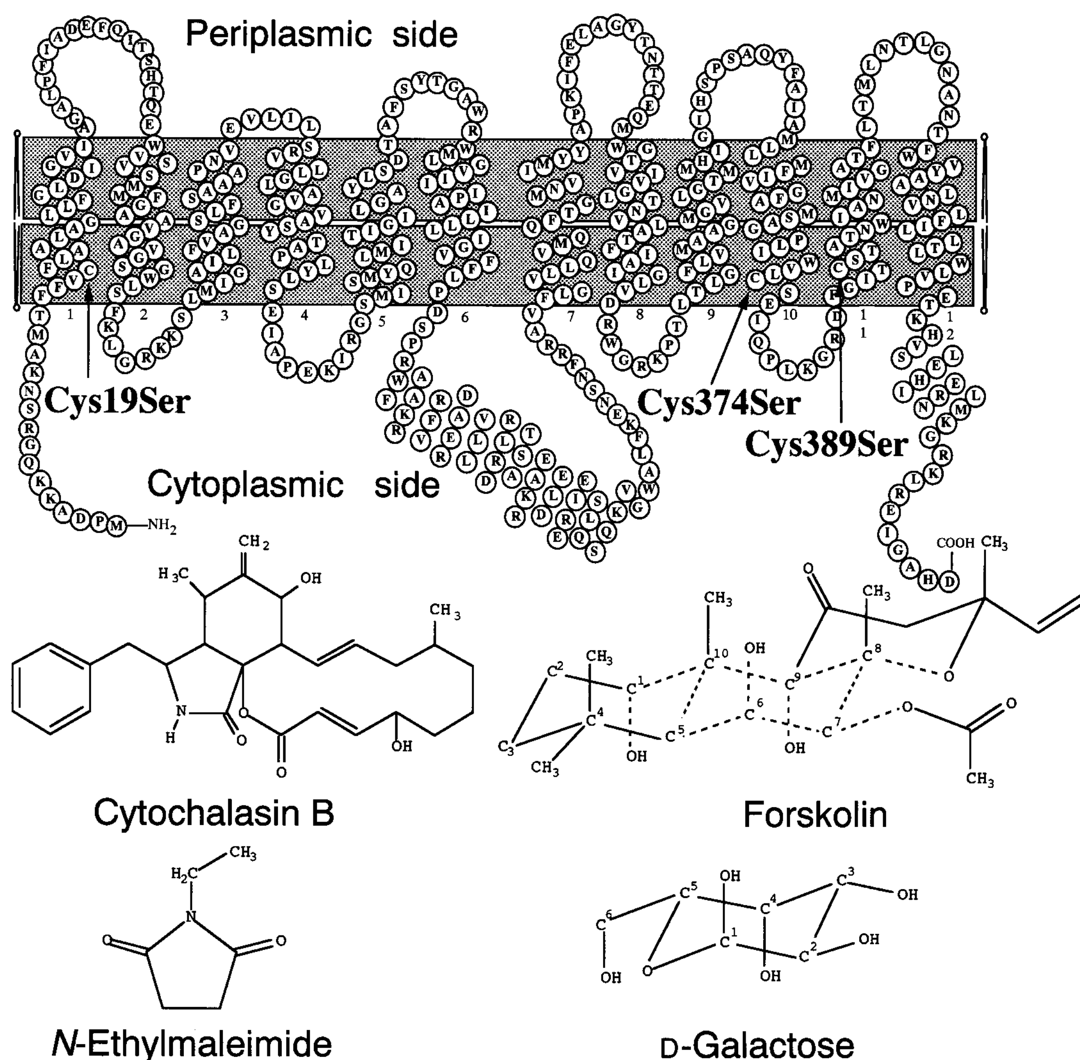

Figure 1 Two-dimensional model of the galactose–H^{+} symport protein

The positions of Cys residues (Cys^{19}, Cys^{374} and Cys^{389}) are indicated by arrows. The structures of the sugar substrate D-galactose, the inhibitory antibiotics cytochalasin B and forskolin, and the thiol-group-reactive reagent NEM are also shown.

galP genes, was the host strain for the overproduction of the GalP protein. After the mutagenic reactions (see below), the recombinant bacteriophage M13 DNA was transfected into *E. coli* strain TG1 $^{\Delta}$(*lac-proAB*) *supE thi hsd*$^{\Delta}$5 F′[*traD*36 *proAB*$^{+}$ *lacI*^{q} *lacZ* ΔM15]. The modification (−) and restriction (−) phenotype of strain TG1 necessitated that the mutant plasmids constructed from DNA isolated from strain TG1 were transformed into strain DH1 [modification (+), restriction (−)] before introduction into *E. coli* strain JM1100 [modification (+), restriction (+) phenotype] to prevent restriction of unmodified plasmid DNA. *E. coli* strain DH1 has the genotype *supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1.

Construction of mutants

Individual codon changes were introduced into the DNA coding for the GalP protein of *E. coli* by oligonucleotide-directed mutagenesis, in which the codon (TGC) for Cys was replaced by that of serine (TCC). Single-stranded DNA template coding for the N-terminal half of GalP was prepared by subcloning a 3.02 kbp *Bam*HI/*Pst*I DNA fragment from the expression plasmid pPER3 into bacteriophage M13. Similarly a template for the C-terminal half was prepared by subcloning a 2.46 kbp *Alw*I/*Alw*I DNA fragment. Mutagenesis was carried out with the Amersham *in vitro* mutagenesis kit using the following mutagenic primers: 5′-GGCAAGGAAGGAGACG-AAAA-3′ (Cys^{19} → Ser); 5′-GAATTTCGGAGGACAGTACC-C-3′ (Cys^{374} → Ser); 5′-GCAGTGGAGGAGGTGATGCCA-3′ (Cys^{389} → Ser) (the mismatches are underlined) complementary to the coding strand of *galP*. The base changes were confirmed by single-stranded dideoxynucleotide sequencing. A 1.78 kbp *Mlu*I/*Bsm*I DNA fragment containing the Cys^{19} → Ser codon change was isolated from the bacteriophage M13 construct and substituted for the wild-type DNA in plasmid pPER3. The codon changes Cys^{374} → Ser and Cys^{389} → Ser were isolated on 1.561 kbp *Mlu*I/*Nde*I DNA fragments and substituted for the wild-type DNA. The substitutions were confirmed by subcloning the DNA containing the mutation back into bacteriophage M13mp18 and dideoxynucleotide sequencing. To construct a *galP* gene with two codon changes (Cys^{19,389} → Ser), a 1.78 kb

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*Bsm*I/*Mlu*I *galP* fragment containing codon Cys^{19} → Ser and a 4.99 kbp *Mlu*I/*Bsm*I *galP* fragment containing codon Cys^{389} → Ser were isolated from plasmid pPER3 and ligated together. DNA from this construct was used in a further reaction using the Cys^{374} → Ser mutagenic oligonucleotide to make a *galP* gene containing no Cys codons. Confirmation that there were no other base changes in the *galP* gene sequence other than those intended was obtained by double-stranded sequencing of the entire gene in all cases.

Growth of *E. coli* strains

The *E. coli* strain JM1100 (pPER3) (wild-type GalP [2]) and JM1100 (plasmids pTPM2, 3, 4, 5 and 10) (GalP with Cys → Ser mutations; the present study) were used for the constitutive overexpression of the wild-type and mutated GalP proteins. To gain maximum expression of GalP, strains were grown overnight in minimal media with 15 μg/ml tetracycline, supplemented with L-histidine (80 μg/ml) and thymine (20 μg/ml). For sugar-transport experiments, requiring lower levels of expression, cells were grown on rich media [2TY (10 g of yeast extract, 10 g of tryptone and 5 g of NaCl made up to1 litre with water) supplemented with 20 mM glycerol, 20 μg/ml thymine and 15 μg/ml tetracycline] into mid-exponential phase ($D_{680} = 0.6$).

Protein assays

The concentration of protein in the membrane preparations was assayed by the method of Schaffner and Weissman [32].

Quantification of expression levels

Membrane samples (30 μg of protein) prepared from cells grown on minimal media, i.e. cells expressing high amounts of GalP, were subjected to SDS/PAGE and the membrane proteins stained with Coomassie Blue. The levels of expression of mutant GalP proteins compared with the wild-type were determined by a comparison of the intensities of the bands corresponding to GalP by scanning with a Molecular Dynamics 100A computing densitometer. The expression levels of the mutant GalP proteins compared with that of the wild-type prepared from cells grown on minimal media are as follows: Cys^{19} → Ser, 66 %; Cys^{374} → Ser, 82 %; Cys^{389} → Ser, 88 %; $Cys^{19,389}$ → Ser, 43 %; $Cys^{19,374,389}$ → Ser, 35 %.

Preparation of membrane vesicles

Vesicles were prepared either from intact cells by explosive decompression in a French press [33] or from spheroplasts [34,35] by the method of Kaback and Barnes [36]. The former procedure yields predominantly inside-out (ISO) vesicles [33,37–39], whereas the latter yields predominantly right-side-out (RSO) vesicles [23,33,36–41]. Where required, inner membranes were isolated from French-press vesicles by sucrose-density-gradient centrifugation essentially as described by Osborn et al. [42].

Labelling proteins with NEM

ISO membrane vesicles (4 mg/ml) were incubated with 500 mM D- or L-galactose for 5 min at 25 °C in 50 mM potassium phosphate buffer (pH 6.6)/10 mM $MgSO_4$. The membranes were allowed to react with 1 mM [*ethyl*-2-^{3}H]NEM (10 μCi/ml) for 15 min at 4 °C and the reaction stopped by the addition of 20 mM 2-mercaptoethanol. The membranes were collected by centrifugation (100000 ***g*** for 1 h at 4 °C) and resuspended into 15 mM Tris/HCl, pH 7.5. The reaction of the proteins with NEM was monitored by SDS/PAGE and fluorography.

Labelling proteins with eosin-5-maleimide

Membrane vesicles (4 mg/ml) were incubated with 500 mM D- or L-galactose for 5 min at 25 °C in 50 mM potassium phosphate buffer (pH 6.6)/10 mM $MgSO_4$. The membranes were then allowed to react with 0.5 mM eosin-5-maleimide in the dark for 15 min at 25 °C and the reaction stopped by the addition of 20 mM 2-mercaptoethanol. The membranes were collected by centrifugation (100000 ***g*** for 1 h at 4 °C) and resuspended into 15 mM Tris/HCl, pH 7.5. The proteins were separated by SDS/PAGE and the labelled proteins revealed by exposing the unstained gel immediately to UV light at 245 nm via a transilluminator.

Sugar-transport measurement

The transport of radioisotope-labelled D-galactose into intact cells was measured after the cells were energized with 10 mM glycerol as described by Henderson et al. [31]. All transport measurements were carried out on cells that were grown on rich media producing levels of GalP expression less than 1 % of total membrane protein. The relative amounts of mutant proteins were determined by Western blots using a polyclonal antibody raised in rabbits against a peptide corresponding to GalP N-terminal sequence. The immune complex was further coupled to swine anti-rabbit IgG–peroxidase conjugate [Dako (Glostrup, Denmark) immunoglobulins] and detected using enhanced chemiluminescence (ECL®; Amersham). The expression level of each mutant protein was shown to be very similar to that of the wild-type protein (results not shown). The steady-state kinetic parameters, apparent K_m and V_{max}, were determined using initial rates of D-[1-^{3}H]galactose transport (at 15 s^{-1}) over an appropriate range of concentrations from about 0.5 K_m to 5 K_m [43]. The values, with their S.D. values, were obtained by a least-squares fit of the unweighted data directly to a hyperbola using the Biosoft program UltraFit [43]. The determination was performed once for each of the mutants and twice for the wild-type. Day-to-day variability of parameter estimations was about 20 %.

Fluorescence studies

Equilibrium fluorescence measurements were measured in a Perkin–Elmer LS50B spectrofluorimeter. Dissociation constants (K_d) for the binding of ligands were determined by a titration of the protein's fluorescence. The protein was excited at 280 nm and the fluorescence emission monitored between 300 and 400 nm. The measurements were carried out in 50 mM potassium phosphate/100 mM NaCl/1 mM EDTA, buffered to pH 7.4 at 20 °C. Routinely, inner membranes were used at a protein concentration of 200 μg/ml in the above buffer. Data were analysed using an unweighted non-linear least-squares fit of the data to a hyperbola using the Biosoft program UltraFit.

Equilibrium dialysis

Equilibrium dialysis was carried out as described by Martin et al. [17]. The binding of [12-^{3}H]forskolin and [4-^{3}H]cytochalasin B to inner membranes (1.0 mg/ml) containing GalP was measured over the range 0.05–80 μM ligand at 4 °C. Ratios of bound to free ligand were calculated from the equilibrium distribution of the radiolabelled ligand and used to determine both the K_d and the number of ligand binding sites by an unweighted non-linear least-squares fit of the data to a hyperbola using the Biosoft program UltraFit [43].

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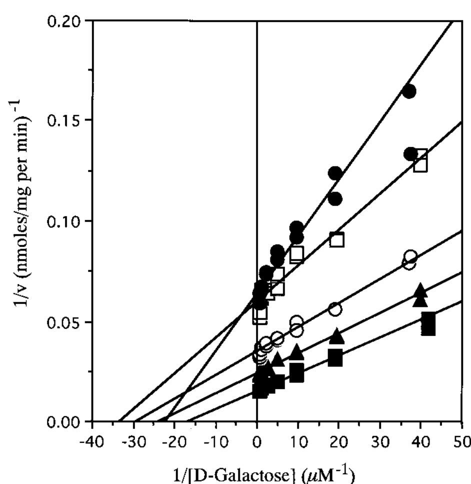

Figure 2 No individual cysteine residue of GalP is essential for energized D-galactose transport

E. coli strain JM1100 expressing the wild-type and mutant proteins were grown in 2TY (see text) supplemented with 20 mM glycerol until D_{680} was 0.6. The cells were harvested and washed twice in the growth volume of 150 mM KCl/5 mM Mes, pH 6.6, and finally resuspended to a density of 0.68 mg of dry cell mass/ml. Aerated samples (0.25 ml) were incubated at 25 °C for 3 min with 25 mM glycerol before addition of 6.25 μl of stock radiolabelled sugar solutions to give the indicated concentration range. After 15 s, 2 ml was filtered rapidly and washed four times in 2 ml of the above medium. The initial rates of sugar uptake (nmol/min per mg of dry mass) were calculated and displayed as Lineweaver–Burk plots. The graph is a typical example of the uptake of D-[1-^{3}H]galactose uptake catalysed by wild-type and mutant proteins. ■, Wild type: $K_m = 42.3 \pm 4.7$ μM; $V_{max} = 59.1 \pm 1.6$ nmol/min per mg; ▲, Cys^{389} → Ser: $K_m = 41.3 \pm 6.2$ μM; $V_{max} = 41.1 \pm 1.4$ nmol/min per mg; ○, Cys^{19} → Ser: $K_m = 34.7 \pm 4.9$ μM; $V_{max} = 28.7 \pm 0.9$ nmol/min per mg; ●, Cys^{374} → Ser: $K_m = 44.6 \pm 9.9$ μM; $V_{max} = 15.5 \pm 0.7$ nmol/min per mg; □, Cys^{19,389} → Ser: $K_m = 30.0 \pm 7.2$ μM; and $V_{max} = 16.7 \pm 0.8$ nmol/min per mg. The mutants were able to accumulate intracellular D-galactose to 23–47-fold that of the external media compared with 47.4–57.7-fold for the wild-type.

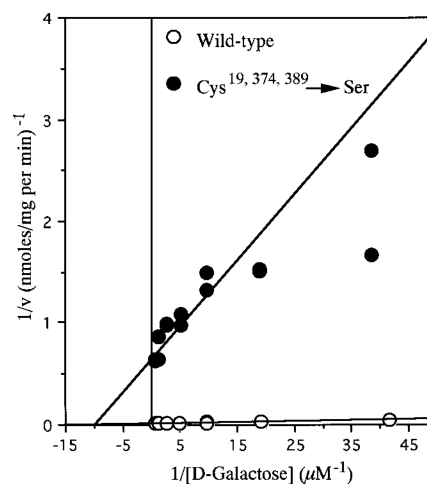

Figure 3 The 'Cys-free' GalP protein (Cys^{19,374,389} → Ser) catalyses energized transport with a K_m similar to that of the wild-type but with a much reduced maximum velocity (V_{max})

E. coli strain JM1100 expressing the wild-type and Cys^{19,374,389} → Ser mutant proteins were grown and the uptake of radiolabelled sugar assayed as described for Figure 3. The initial rates of sugar uptake (nmol/min per dry mass) were calculated and displayed as a Lineweaver–Burk plot. The graph is a typical example of the uptake of D-[1-^{3}H]galactose catalysed by the wild-type and the Cys^{19,374,389} → Ser mutant proteins. The data yielded K_m values of 58.9 ± 8.6 and 100 ± 44.1 μM and V_{max} values of 65.4 ± 2.3 and 1.54 ± 0.19 nmol/min per mg for wild-type and mutant respectively. The Cys^{19,374,389} → Ser protein was able to accumulate intracellular D-galactose to 7-fold that of the external media compared with 47.4–57.7-fold for the wild-type.

RESULTS

Energized D-galactose transport catalysed by GalP with single Cys → Ser substitutions is only slightly impaired

The transport of D-galactose catalysed by the wild-type GalP protein, under energized conditions, was characterized by an hyperbolic increase in the initial rate with increasing concentration of sugar. A least-squares fit yielded apparent K_m and V_{max} values of 42.4–58.9 μM and 62.8–65.4 nmol/min per mg (Figures 2 and 3). K_m and V_{max} values are apparent, as it is not known whether the co-substrate, H^+, is at saturating amounts. It is assumed that the Δp, the transmembrane electrical potential, is unaffected by the low levels of GalP expression operating in these experiments (see the Materials and methods section).

The K_m values for D-galactose transport (which are a function of the rate constants for both substrate binding and translocation), catalysed by the Cys^{19} → Ser, Cys^{374} → Ser and the Cys^{389} → Ser GalP mutants were similar to that of the wild-type protein. In contrast with the K_m values, the V_{max} values (a function largely of the rate constants for translocation) are decreased compared with the wild-type (Figure 2). All the mutant proteins were able to mediate energized accumulation of D-galactose against the concentration gradient, showing that none of the Cys residues are uniquely essential for transport. However, substitution of the Cys^{374} residue (Cys^{374} → Ser) caused the biggest reduction (4.2-fold) in activity as defined by V_{max}/K_m. This comparatively severe effect of changing the Cys^{374} residue was further demonstrated by data showing that the GalP protein containing only Cys^{374} (Cys^{19,389} → Ser) was able to catalyse D-galactose transport with only a 2.2-fold reduction in activity. The GalP protein with no Cys residues (Cys^{19,374,389} → Ser) was able to accumulate D-galactose against the concentration gradient and transport exhibited a K_m that was only 2-fold higher than the wild-type, but the V_{max} was drastically reduced, being 40-fold lower (Figure 3).

It seems that the three Cys substitutions in combination have a greater deleterious effect on GalP than the sum of each of the individual mutations.

Only Cys^{374} of GalP reacts with NEM

Inner-membrane French-press vesicles from *E. coli* strain JM1100 overexpressing either the Cys^{19} → Ser, Cys^{374} → Ser, Cys^{389} → Ser or the wild-type GalP proteins were prepared (see the Materials and methods section). The membrane samples were allowed to react with 1 mM [*ethyl*-2-^{3}H]NEM in the presence of either D- or L-galactose (D-galactose is the physiological substrate, whereas L-galactose is not a substrate). The membrane samples were separated by SDS/PAGE and the reaction with *N*-[*ethyl*-2-^{3}H]ethylmaleimide monitored by fluorography (Figure 4). The wild-type GalP protein and the mutants Cys^{19} → Ser and Cys^{389} → Ser reacted with NEM, and the reaction was protected by D-galactose. The Cys^{374} → Ser mutant protein did not react with NEM. [In some preparations, but not others, there was radiolabelling of a minor protein migrating just above 43 kDa (Figure 4). Its appearance in the control membranes without GalP present, and the absence of protection by sugar, show it has no relation to the conclusions herein.]

These experiments indicate that GalP contains a single Cys residue available for the substrate-protectable reaction with NEM and that this residue is Cys^{374}.

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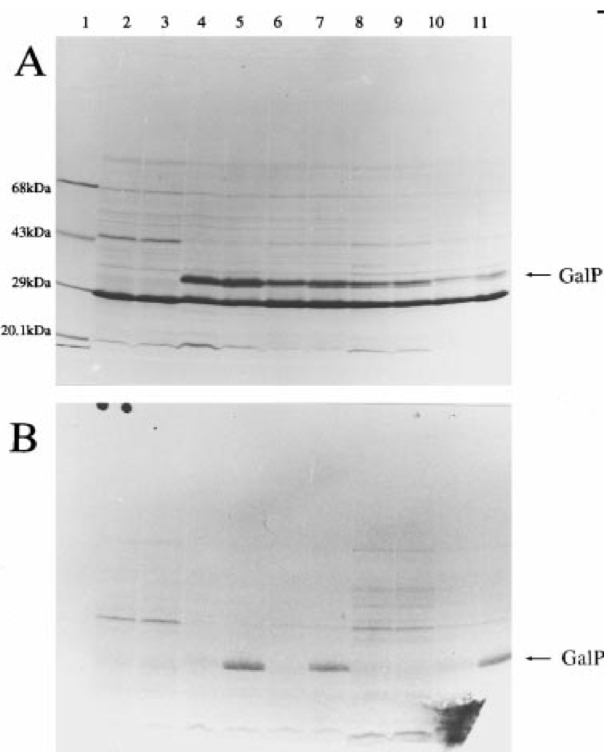

Figure 4 Only Cys^{374} of GalP reacts with NEM

ISO membrane vesicles were prepared from *E. coli* strain JM1100 overexpressing the wild-type and mutant proteins using a French press as described in the Materials and methods section. The protein samples, together with one expressing no GalP as a negative control, were allowed to react with 1 mM [*ethyl*-2-^{3}H]NEM in the presence of either 500 mM D- or L-galactose. The proteins (30 μg) were loaded on an SDS/12.5%-polyacrylamide gel as indicated, separated, and then stained with Coomassie Blue (**A**). The characteristic appearance of GalP proteins at 34–36 kDa is indicated by the arrow. The gel was dried and the reaction of GalP with [*ethyl*-2-^{3}H]NEM was monitored by fluorography (**B**). Lane 1, molecular-mass standards; lane 2, negative control (membranes prepared from *E. coli* JM1100 carrying plasmid pBR322) plus D-galactose; lane 3, negative control plus L-galactose; lane 4, wild-type plus D-galactose; lane 5, wild-type plus L-galactose; lane 6, Cys^{19} → Ser plus D-galactose; lane 7, Cys^{19} → Ser plus L-galactose; lane 8, Cys^{374} → Ser plus D-galactose; lane 9, Cys^{374} → Ser plus L-galactose; lane 10, Cys^{389} → Ser plus D-galactose; lane 11, Cys^{389} → Ser plus L-galactose.

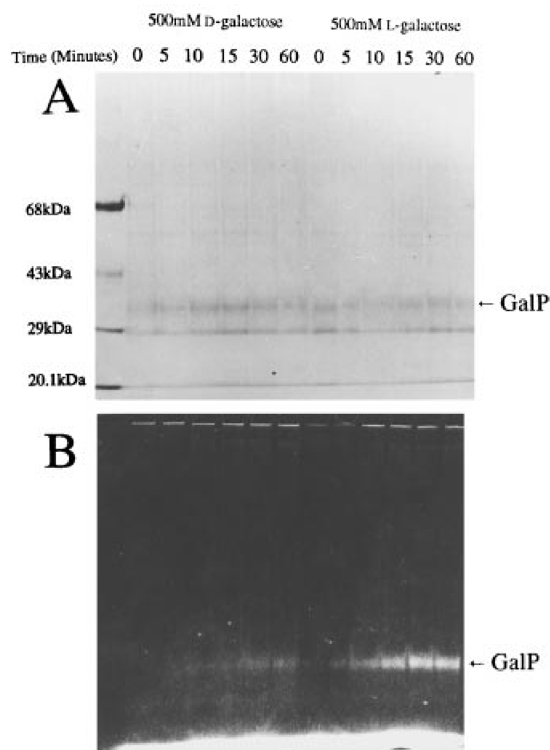

Figure 5 Cys^{374} reacts with eosin-5-maleimide in a manner protected by substrate

ISO membrane vesicles were prepared from *E. coli* strain JM1100 containing the Cys^{19,389} → Ser mutant *galP* plasmid grown on minimum medium as described in the Materials and methods section. The membranes were allowed to react with 0.5 mM eosin-5-malemide in the presence of 500 mM D- or L-galactose and the reaction stopped at timed intervals) (see the Materials and methods section). The protein samples were separated by electrophoresis on an SDS/12.5%-polyacrylamide gel and the unstained gel viewed under UV light at 245 nm (**B**) (see the Materials and methods section). The gel was then stained with Coomassie Blue (**A**).

The mutant containing only one Cys residue, Cys^{374}, of GalP reacts with eosin-5-maleimide in a substrate-protectable manner

To use the thiol-group-specific reagent eosin-5-maleimide as a topological probe of GalP it was necessary to determine first if it reacts with Cys^{374} and if the reaction is protected by substrate. The incorporation of eosin-5-maleimide into proteins can be easily monitored, since the compound emits fluorescence under UV light. ISO membrane vesicles prepared from *E. coli* JM1100 expressing the Cys^{19,389} → Ser mutant protein were labelled with 0.5 mM eosin-5-maleimide in the presence of 500 mM D- or L-galactose and samples taken and quenched at intervals up to 60 min. The labelled protein samples were size-fractionated by SDS/PAGE as already described, and the unstained gel was viewed on a UV transilluminator at 245 nm (Figure 5). The GalP protein clearly exhibited more intense fluorescence in the set of samples labelled in the presence of L-galactose than in the set labelled in the presence of D-galactose (Figure 5).

This experiment confirms that only Cys^{374} reacts with the thiol-group-specific reagent, and that this reaction is protected specifically by D-galactose. The reaction with the hydrophilic reagent eosin-5-maleimide suggests that Cys^{374} is in a hydrophilic rather than a hydrophobic environment, which is expected if Cys^{374} is in or near the sugar-binding site or pocket of the protein.

The differential reaction of GalP with eosin-5-maleimide in ISO and RSO vesicles indicates that Cys^{374} is exposed to the cytoplasmic side of the membrane

To investigate the topology of Cys^{374} with respect to the membrane, eosin-5-maleimide was tested for its reaction with GalP in ISO and RSO vesicles. Membrane vesicles were prepared (see the Materials and methods section) that contained the Cys^{19,389} → Ser GalP protein. SDS/PAGE was used to show that both sets of vesicles contained the same amount of GalP (results not shown). The membrane vesicles were allowed to react with eosin-5-maleimide in the presence of 500 mM L-galactose for 0, 5, 10, 15, 30 and 60 min, and the labelled proteins were size-fractionated by SDS/PAGE. The extent of reaction was measured by monitoring the fluorescence produced by irradiation with UV light, which showed that the eosin-5-maleimide reacted more readily with Cys^{19,389} → Ser GalP protein in the ISO vesicles than the RSO vesicles over the 60 min period (Figure 6). It was also observed that the GalP band in the ISO vesicles is much more diffuse than that in the RSO vesicles; this is presumably

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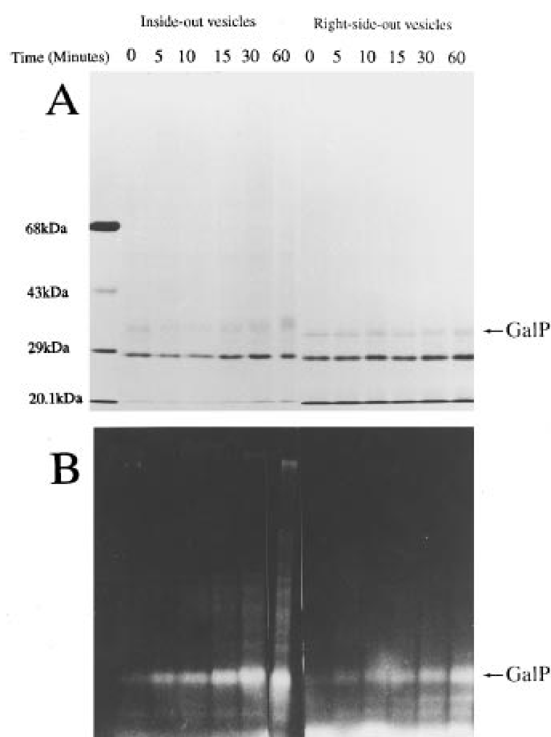

Figure 6 Reaction between eosin-5-maleimide and Cys³⁷⁴ of the GalP protein occurs more readily in ISO than RSO membrane vesicles

ISO French press and RSO membrane vesicles were prepared from *E. coli* strain JM1100 overexpressing GalP containing only the eosin-5-maleimide reactive Cys residue, Cys^{374} ($Cys^{19,389} \rightarrow$ Ser). The membrane vesicles, containing identical amounts of GalP, were allowed to react with 0.5 mM eosin-5-maleimide for the indicated time. The proteins were separated on an SDS/12.5%-polyacrylamide gel and the reaction of GalP with eosin-5-maleimide monitored by viewing the fluorescence of the unstained gel irradiated with light at 245 nm (**B**) and the proteins were then stained with Coomassie Blue (**A**). The characteristic appearance of the GalP protein at 34–36 kDa is identified by the arrows. The example shown is typical of three completely independent experiments using different membrane preparations, in which GalP in the ISO vesicles always reacted more readily than did that in the RSO vesicles.

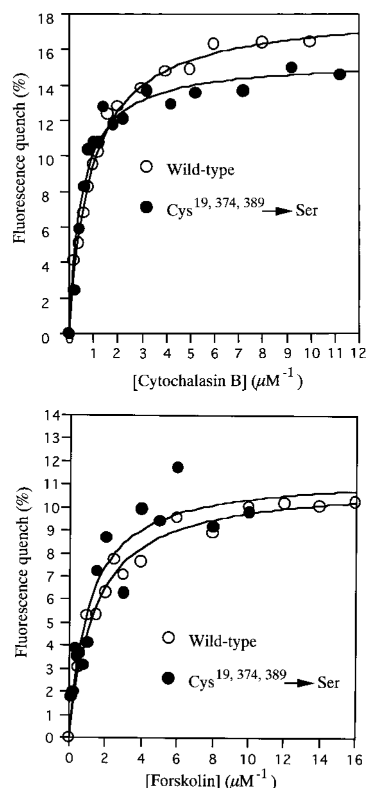

Figure 7 'Cys-free' protein binds the antibiotics cytochalasin B and forskolin with K_d values similar to those of the wild-type

The graphs show the concentration-dependence of the total fluorescence quench of $Cys^{19,374,389} \rightarrow$ Ser and wild-type proteins ($\lambda_{excitation}$ 280 nm; $\lambda_{emission\,(max)}$ 330–340) induced by the binding of cytochalasin B (upper panel) and by forskolin (lower panel). The data, when fitted by the least-squares method to a hyperbolic equation, yielded overall dissociation constants (K_d) for forskolin binding of 1.39 ± 0.33 μM (wild-type 1.48 ± 0.56 μM) and for cytochalasin B binding of 0.91 ± 0.12 μM (wild-type 1.1 ± 0.19 μM).

because more of the protein is modified by the reaction with eosin-5-maleimide. This experiment was carried out three times using different vesicle preparations and each time gave similar results. Some variation in the representative orientation of the vesicles within each preparation may explain why GalP reacts with eosin-5-maleimide in both preparations to some extent.

These data suggest that the reactive Cys^{374} residue is exposed to the inside of the cell.

The 'Cys-free' protein ($Cys^{19,374,389} \rightarrow$ Ser) binds both the antibiotics cytochalasin B and forskolin with K_d values similar to that of the wild-type

It has been shown that the rate of D-galactose transport catalysed by GalP devoid of Cys residues ($Cys^{19,374,389} \rightarrow$ Ser) is severely impaired, as defined by the V_{max} (although the K_m is relatively unaffected). To investigate the possibility that the gross conformation of the protein had been disrupted, K_d values for the binding of cytochalasin B and forskolin were determined by measuring the quench in the intrinsic fluorescence of the mutant protein induced by the binding of the antibiotics [16–18]. The intrinsic fluorescence ($\lambda_{excitation}$ 297 nm; and $\lambda_{emission,max.}$ 330–340nm) was titrated with antibiotic over a range of concentrations up to 80 μM (Figure 7). A fit of the data to an hyperbola yielded an overall dissociation constant for forskolin binding of 1.39 ± 0.33 μM (wild-type 1.48 ± 0.56 μM) and for cytochalasin B binding of 0.91 ± 0.12 μM (wild-type 1.1 ± 0.19 μM). The similarities of the K_d values for the binding of the antibiotics to both the wild-type and the mutant proteins shows that both the antibiotic binding sites are conserved in the Cys-free GalP protein, suggesting that the gross conformation of the protein is maintained, despite the change in V_{max} (above).

We conclude that no individual Cys residue is an absolute requirement for maintaining the binding site of cytochalasin B or forskolin.

All the 'Cys-free' GalP protein in the membrane is folded correctly, as defined by the binding of forskolin

The conservation of antibiotic binding sites (see above) suggests that the 'Cys-free' protein is folded correctly in the membrane. However, it is possible that only a small percentage of the expressed protein is folded in a way that can bind antibiotic. To address this possibility, equilibrium binding of radioisotopically labelled forskolin [17,18] was used to measure the concentration of binding sites in the membrane; the equilibrium binding of forskolin to GalP is characterized by a linear Scatchard plot, indicating a single species of high-affinity binding sites [17]. Data from equilibrium binding of forskolin to the $Cys^{19,374,389} \rightarrow$ Ser

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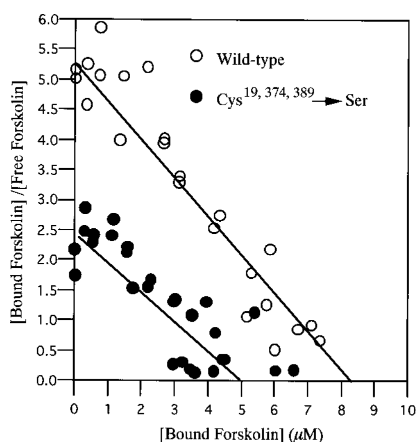

Figure 8 All the 'Cys-free' GalP protein expressed in the membrane is folded correctly as defined by the binding of forskolin

Inner-membrane vesicles containing either over-expressed Cys^{19,374,389} → Ser or wild-type proteins were subjected to microdialysis against a range of forskolin concentrations (0.05–80 μM), and the proportion of forskolin bound to the protein and free in solution were measured using [12-^{3}H]forskolin. The data were analysed by a least-squares fit to a hyperbola from which the overall K_d values of 1.48 ± 0.78 and 1.36–1.43 μM and the number of binding sites to be 4.9 ± 0.65 and 8.3 ± 2.9 nmol/mg of membrane protein for mutant and wild-type respectively. These values, when compared with the amount of expressed mutant and wild-type GalP determined by quantitative densitometry of SDS/PAGE gels, indicated an approx. 1:1 ratio for the binding of forskolin to the proteins.

mutant GalP and wild-type proteins were fitted to a single hyperbolic function by least-squares analysis [43], and K_d values for forskolin binding of 1.48 ± 0.78 and 1.36–1.43 μM for the mutant and wild-type respectively were revealed (Figure 8). These data corroborate those obtained from fluorescence-quenching experiments. The number of binding sites were measured at 4.9 ± 0.65 and 8.3 ± 2.9 nmol/mg of membrane protein for mutant and wild-type respectively. These values were compared with the amount of expressed GalP as determined by quantitative densitometry of SDS/PAGE gels (see the Materials and methods section), and indicated approximately a 1:1 ratio for the binding of the antibiotics to the mutant and the wild-type protein. The equimolar relationship between the number of forskolin-binding sites and the amount of protein in the membrane demonstrates that a conformation capable of binding forskolin is undertaken by all of the Cys-free protein in the cytoplasmic membrane.

DISCUSSION AND CONCLUSIONS

Our data show that the GalP protein with Cys^{374} substituted with Ser did not react with the thiol-group-specific reagent NEM, whereas the GalP proteins with Ser substitutions at Cys^{19} and Cys^{389} continued to react normally with NEM and the reactions were prevented by the addition of the substrate, D-galactose. These results show that Cys^{374} is the only residue accessible for modification by NEM, and suggest that Cys^{19} and Cys^{389} are protected from reaction by the three-dimensional structure of the protein. The protection by substrate of Cys^{374}, which is predicted in a two-dimensional model of GalP to be at the cytoplasmic side of TM10 (Figure 1), suggests that the residue is near the sugar-binding site. It cannot, however, be ruled out that sugar binding inhibits the modification of Cys^{374} by NEM due to conformational changes with long-range secondary effects; this effect would have to be reciprocated as the reaction with NEM also inhibits the transport of sugar [26].

All the single Cys → Ser mutant proteins were able to transport D-galactose against the concentration gradient, and the K_m parameters were essentially unchanged. However, the V_{max} parameters, which are governed largely by the rate constants for translocation, were diminished. These data indicate that the sugar-binding sites are still intact, but the substitutions hinder the conformational changes involved in sugar translocation, rendering the process less efficient; also, we can conclude that no individual Cys residue is absolutely required to maintain the catalytic activity of GalP. It is therefore unlikely that any Cys residue forms a binding interaction with sugar, which is also confirmed by the lack of any conservation of Cys residues throughout the family of sugar transporters. However, in GalP the rate of transport was most impaired by the mutation of the Cys^{374} residue, and it seems reasonable that, if Cys^{374} is near the sugar-binding site, as suggested by sugar protecting against NEM labelling, its substitution may have a significant effect on the translocation mechanism. The Cys^{19,374,389} → Ser triple-mutant protein was able to catalyse D-galactose transport with a relatively unimpaired K_m, but the V_{max}, even when compared with the other mutant proteins, was drastically reduced. It seems that, combined, the subtle perturbation of the protein by each mutation has a synergistic deleterious effect on the translocation mechanism, but not the sugar binding, of the protein. The maintenance of the gross conformation of the protein is confirmed by equilibrium binding experiments showing that cytochalasin B and forskolin bind to the mutant protein with K_d values similar to those of the wild-type. These data also show that no single Cys residue is required to maintain the binding site of cytochalasin B or forskolin.

To make a complete turn of an α-helix the polypeptide backbone must traverse 3.6 amino acids, so that amino acids three or four amino residues apart would be expected to project from the same side of an α-helix; since Trp^{371} is three amino acids away from Cys^{374}, it would project from the same side of the α-helix (TM10). It was shown in a previous study [44] that the substitution of Trp^{371} with Phe greatly reduced the affinity for sugar of the inward-facing sugar-binding site, leading to the proposal that Trp^{371} is involved in the interaction with sugar. This observation provides further circumstantial evidence that Cys^{374} is near the internal sugar-binding site. The extensive reaction of the Cys^{19,389} → Ser GalP mutant (and of the wild-type; results not shown) with the membrane-impermeant thiol-directed reagent eosin-5-maleimide suggests that Cys^{374} is in an aqueous environment, as may be expected if Cys^{374} is in or near the binding site or pocket of the protein. This reaction occurred more readily with the mutant protein in ISO vesicles than RSO vesicles, indicating that Cys^{374} is exposed to the inside of the cell and is consistent with the two-dimensional model of GalP (Figure 1), showing the residue to be at the cytoplasmic side of TM10. It has also been shown [44,45] that Trp^{395} and Asn^{394}, of TM11, are associated with the internal binding site for cytochalasin B (both Trp^{395} and Asn^{394}) and D-galactose (only Asn^{394}). We therefore propose that Trp^{371}, Cys^{374}, Asn^{394} and Trp^{395} are directed between TM10 and TM11 to form part of an inward-facing aqueous channel and binding pocket for sugar and antibiotic (Figure 9). We predict from helical-wheel plots, that Cys^{389}, which does not react with NEM, is on the opposite side of TM11 to Asn^{394} and Trp^{395} and does not project into the channel (Figure 9). It has also been concluded that TM10 and 11 of GLUT1 are involved in forming the internal binding sites for sugar and antibiotics [46–50], providing further evidence of the structural similarity of GLUT1 and GalP.

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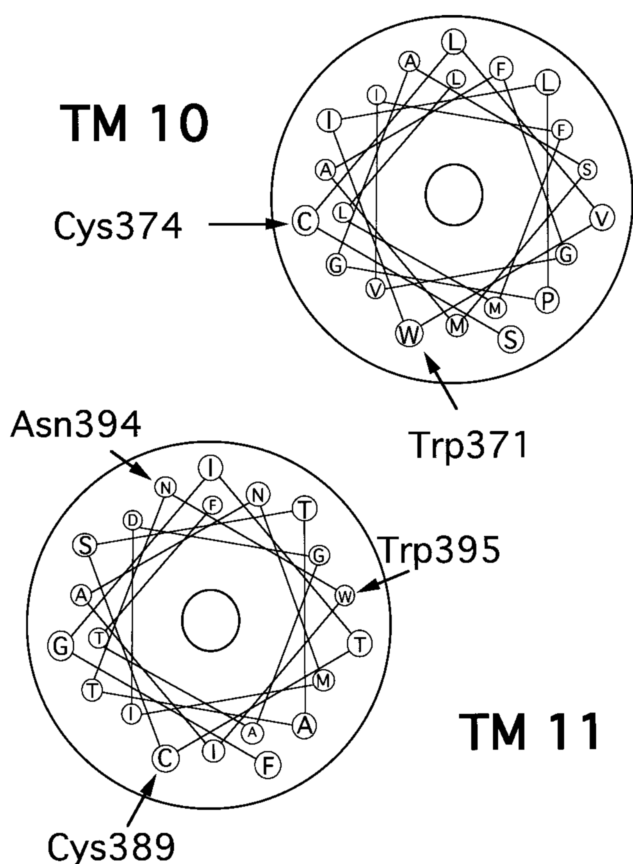

Figure 9 Helical-wheel model of helices 10 and 11 in GalP

It is common for membrane transport proteins to be inhibited by thiol-group-reactive reagents, and for the reaction to be attenuated by substrates. Examples from the Multi Facilitator Superfamily include the arabinose–H^+ [24], xylose–H^+ [25] and lactose–H^+ (see, e.g., [51–53]) symporters of *E. coli*, and GLUT1 itself (reviewed in [54]). In all cases examined, as with GalP, the reactive cysteine residues are not conserved in sequences of aligned family members; also, only a minority, usually one, cysteine out of several seems accessible to NEM in an aqueous pocket in the protein, and Cys-free mutants retain their structure–activity relationship. The Cys-free mutants are therefore useful for subsequent genetical/biochemical manipulations to elucidate the molecular mechanism, as illustrated particularly by studies on LacY and GLUT1 (see, e.g., [53–55]). The fact that some transport proteins are not inhibited by maleimides, e.g. the L-fucose–H^+ and L-rhamose–H^+ transporters [56] does not rule out the utility of Cys mutagenesis for analysing their structure–activity relationships.

The low-resolution model for GalP described above (and Figure 9) should be considered as a base for refined experimentation to determine the proximity of residues to each other and the bound sugar more precisely. Our construction of the 'Cys-free' GalP protein, which is functional in terms of both sugar and antibiotic binding, provides the means to achieve this, since it can serve to reintroduce Cys residues back into GalP at any defined location. Such constructs could be used for investigations using thiol-specific fluorescent probes such as *N*-(1-pyreneyl)maleimide [53]. Other approaches could include the use of site-directed cross-linking between inserted Cys pairs and Cys-scanning mutagenesis [53]. The reintroduction of Cys residues could also be used in conjunction with ESR labels, or solid-state NMR combined with magic-angle spinning, to obtain structural information [57,58].

This work was supported by research grants from the Wellcome Trust, the Medical Research Council, the Biotechnology and Biological Sciences Research Council (BBSRC), the European Union and by equipment grants from the University of Leeds, and was partly conducted in the Astbury Centre for Structural Molecular Biology within the North of England Structural Biology Consortium (NESBIC), funded by BBSRC. We are grateful to Mr J. O'Reilly for isolating the inner-membrane preparations from *E. coli* and Ms D. Ashworth for oligonucleotide synthesis and DNA sequencing.

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Received 16 August 2000/25 October 2000; accepted 17 November 2000

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