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Specific spin labelling of the sugar-H^+ symporter, GalP, in cell membranes of *Escherichia coli*: site mobility and overall rotational diffusion of the protein

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Abstract

The D-galactose-H^+ symport protein (GalP) of *Escherichia coli* is a homologue of the human glucose transport protein, GLUT1. After amplified expression of the GalP transporter in *E. coli*, other membrane proteins were prereacted with *N*-ethylmaleimide in the presence of excess D-galactose to protect GalP. Inner membranes were then specifically spin labelled on Cys^{374} of GalP with 4-maleimide-2,2,6,6-tetramethylpiperidine-1-oxyl. The electron paramagnetic resonance (EPR) spectra are characteristic of a single labelling site in which the mobility of the spin label is very highly constrained. This is confirmed with other nitroxyl spin labels, which are derivatives of iodoacetamide and indanedione. Saturation transfer EPR spectra indicate that the overall rotation of the GalP protein in the membrane is slow at low temperatures (approx. 2°C), but considerably more rapid and highly anisotropic at physiological temperatures. The rate of rotation about the membrane normal at 37°C is consistent with predictions for a 12-transmembrane helix assembly that is less than closely packed.

Keywords: Membrane protein; Glucose transport protein 1; Sugar transport

1. Introduction

The D-galactose-H^+ membrane transport protein (GalP) is the hexose-specific member of a family of sugar-H^+ symporters that are responsible for active uptake of sugars by *Escherichia coli* [1–3]. These bacterial transporters are homologous to the GLUT family of mammalian passive facilitated glucose transporters [4]. In particular, GalP possesses a sugar specificity very similar to that of the GLUT1 transporter, and is similarly inhibited by the antibiotics forskolin and cytochalasin B. Although three-dimensional crystal structures are not available, both

Abbreviations: 6-MSL, 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl; IASL, 4-(iodoacetamido)-2,2,6,6-tetramethylpiperidine-1-oxyl; 5-InVSL, 2-[(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)methenyl]indane-1,3-dione; NEM, *N*-ethylmaleimide; EPR, electron paramagnetic resonance; ST-EPR, saturation transfer EPR; V_1 display, first harmonic, absorption EPR spectrum recorded in-phase with respect to the magnetic field modulation; V_2' display, second harmonic, absorption ST-EPR spectrum recorded 90° out-of-phase with respect to the magnetic field modulation

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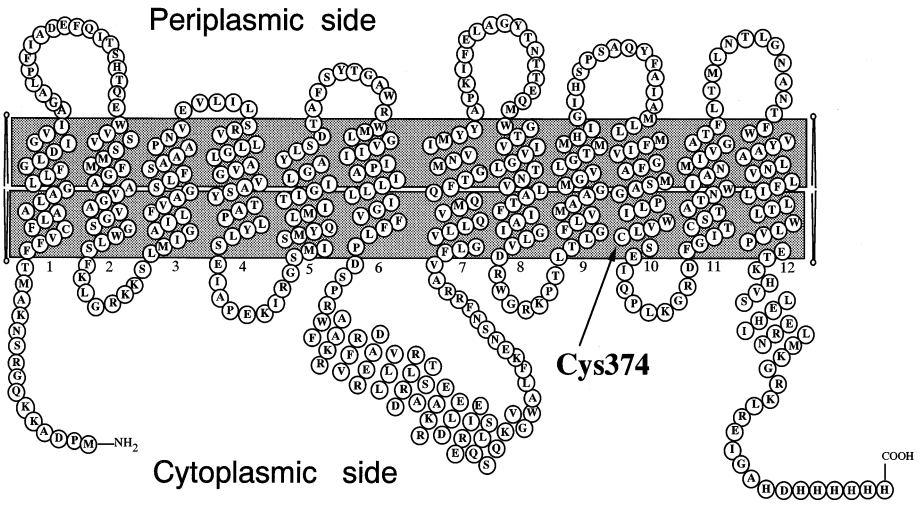

Fig. 1. Transmembrane topography of GalP predicted from the amino acid sequence. The protein contains three cysteine residues. Only Cys^{374} on the tenth predicted transmembrane helix (TM10) is labelled by *N*-ethylmaleimide, in the membranous form.

families are predicted to possess a transmembrane topology that is characterised by 12 membrane-spanning α-helices (see Fig. 1) [3].

Biophysical studies on the bacterial sugar symporters have been made possible by overexpression in *E. coli*. Using a plasmid containing the *galP* gene under control of its own promoter, the level of GalP can be amplified such that it constitutes 50–55%, or more, of the total cytoplasmic membrane protein [5,6]. Specific labelling of the GalP protein can then be achieved in native membranes, after prereacting with a sulphydryl alkylating agent in the presence of excess substrate [7]. Alkylation by *N*-ethylmaleimide, in the absence of substrate, rapidly inactivates the *E. coli* sugar symporters [1]; for GalP, this inactivation is protected to a great extent by 20 mM D-galactose. Site-directed mutagenesis studies have identified the cysteine residue responsible for blocking activity on labelling as the conserved Cys^{374} in the tenth putative transmembrane helix [7]. These studies also showed that, of the three cysteine residues in GalP (see Fig. 1), Cys^{374} is the only one in the folded protein that is modified by NEM.

In the present work, we have conducted EPR studies on GalP overexpressed in plasma membrane vesicles by spin labelling the cysteine residue that blocks activity on chemical modification. Three different spin-labelled alkylating agents are used to assess the local mobility near the active site, and non-linear (saturation transfer) EPR methods are used to determine the overall rotational diffusion rate of the labelled protein in its native environment.

2. Materials and methods

2.1. Materials

Glucose-free D-galactose and NEM were from Sigma (St. Louis, MO, USA). Of the various spin-labelling reagents, 6-MSL (4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl) was from Institut Josef Stefan (Ljubljana, Slovenia), 5-InVSL (2-[(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)methenyl]indane-1,3-dione) was prepared by Prof. K. Hideg (University of Pécs, Hungary) [8], and IASL (4-(iodoacetamido)-2,2,6,6-tetramethylpiperidine-1-oxyl) was from Syva (Palo Alto, CA, USA).

2.2. Preparation of GalP membranes

E. coli strain JM1100 (pPER3), which overexpresses GalP, was grown on minimal medium as described [5,6]. Inside-out plasma membrane vesicles were prepared by explosive decompression of intact cells in a French press [9], and purified on a sucrose density gradient [10].

2.3. Spin labelling

Spin label alkylation of cysteine residues, with or without prereaction with NEM, was carried out essentially as described [1]. Membranes (approx. 1 mg protein) were suspended in 0.5 ml of 50 mM potassium phosphate, 10 mM magnesium sulphate, pH 6.6. For prereaction of other cysteine residues with sulphydryl reagent, the membrane suspension was preincubated with 20 mM D-galactose for 5 min at 25°C, and then incubated with 1 mM NEM for 15 min at 25°C. After incubation on ice for 0.5 min, the membrane suspension was centrifuged for 5 min at full speed in an Eppendorf centrifuge at 4°C. Membranes were then washed three times in 0.5 ml of the same buffer. Prereacted or non-prereacted membranes were incubated with 1 mM spin label reagent, added in 25 μl concentrated ethanol solution, for 15–20 min (30 min for IASL) at 25°C. The membranes were then centrifuged and washed as above. The final membrane pellet was resuspended in a minimal volume of buffer and transferred to a 1 mm ID glass capillary for EPR measurements. The sample was packed in the capillary by centrifugation on a bench-top centrifuge, excess supernatant removed, and the capillary sealed.

2.4. Electron paramagnetic resonance spectroscopy

EPR spectra were recorded on a Varian Century Line 9 GHz spectrometer equipped with gas flow temperature regulation. The sample capillaries were accommodated in a standard 4 mm quartz EPR tube that contained light silicone oil for thermal stability. Temperature was measured with a fine wire thermocouple located in the silicone oil at the top of the microwave cavity. Conventional EPR spectra were recorded in the standard in-phase, first harmonic, absorption V_1 mode. Saturation transfer (ST) EPR spectra were recorded in the 90° out-of-phase, second harmonic, absorption V_2' mode, with a microwave magnetic field at the sample of $\langle H_1^2 \rangle^{1/2} = 0.25$ G. A standardised recording protocol and sample geometry were used for ST-EPR measurements [11,12]. Normalised ST-EPR intensities were calculated as defined in [13]. ST-EPR spectral calibrations of isotropic rotational correlation times were taken from the same reference (see also [14]).

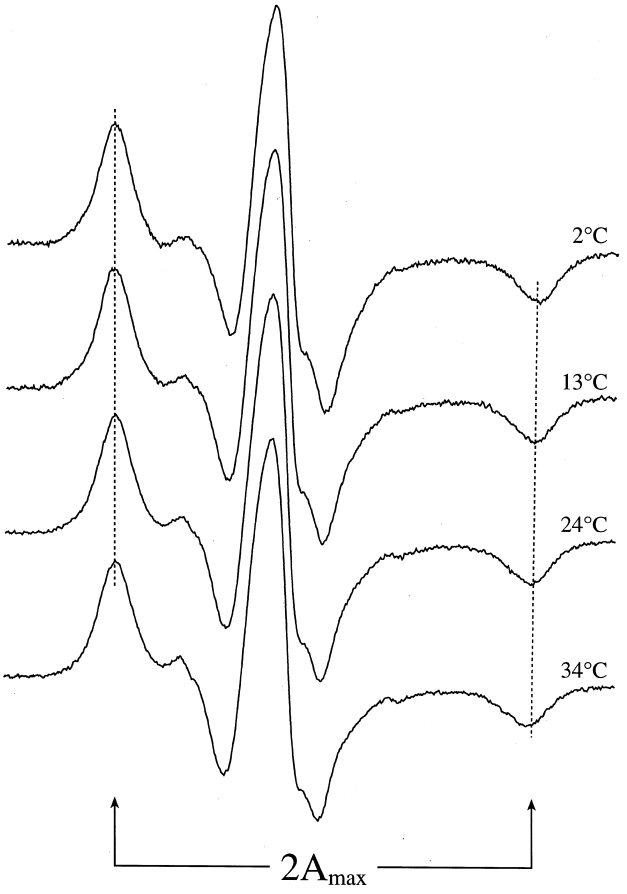

Fig. 2. Conventional EPR spectra (V_1 display) of GalP membranes spin labelled with 6-MSL, after prereacting with NEM in the presence of 20 mM D-galactose. The temperatures at which the spectra were recorded are indicated in the figure. Buffer: 50 mM potassium phosphate, 10 mM magnesium sulphate, pH 6.6. The outer hyperfine splitting, $2A_{max}$, is indicated in the figure. Total scan width = 10 mT.

3. Results

3.1. Conventional EPR spectra

The conventional first harmonic, in-phase, absorption V_1 EPR spectra of GalP membranes spin labelled with 6-MSL, after prereacting with NEM in the presence of 20 mM D-galactose, are shown in Fig. 2. The spectra correspond to a single, homogeneous population of spin labels that are strongly immobilised on the nanosecond time scale, which corresponds to the region of motional sensitivity of conventional spin label EPR. This is consistent with labelling at a specific site that is protected

from labelling by the presence of excess D-galactose, and blocks sugar transport activity on chemical modification (see [1]). The spectral line shapes at low temperature (2°C) are approaching those of the rigid limit for conventional nitroxide EPR spectroscopy, as is also seen from the fact that they change relatively little with increasing temperature. The specific site of spin labelling therefore represents a rather constrained environment.

The V_1 EPR spectra of GalP membranes spin labelled with 6-MSL, 5-InVSL or IASL, without prereaction with NEM, are shown in Fig. 3. Spectra are presented for a low (approx. 2°C) and a high (approx. 37°C) temperature for each spin label. The spectra of 6-MSL contain a small sharp component that is indicated by the arrows labelled 'm' in Fig. 3. This corresponds to labelling at a small fraction of mobile sites and is absent from the spectra of membranes prereacted with NEM (cf. Fig. 2). Otherwise, the major part of the spectra resembles rather closely that of NEM-prereacted membranes that are specifically labelled with 6-MSL, although there are small quantitative differences, as will be seen later. The intensity of spin labelling is greater in the absence of prereaction with NEM. Specific labelling of the protected site corresponds to approx. 30–40% of the total labelling intensity without protection, and is consistent with a single cysteine (Cys^{374}) on GalP being the primary site of labelling in GalP membranes [7] but the protection by D-galactose being only partial [1].

The spectra of 5-InVSL contain three very sharp lines (labelled 'f' in Fig. 3) that can be attributed to free spin label in solution, because labelling with this class of modification reagents can be partially reversible (cf. [8]). The majority of the spectral intensity of 5-InVSL (i.e., the broad component of large spectral extent) corresponds to spin labels that are strongly immobilised, as can be expected from the short and rather rigid link of this vinyl ketone class of reagents, and the steric bulk of the remainder of the molecule [15,16]. There is, however, evidence of an additional,

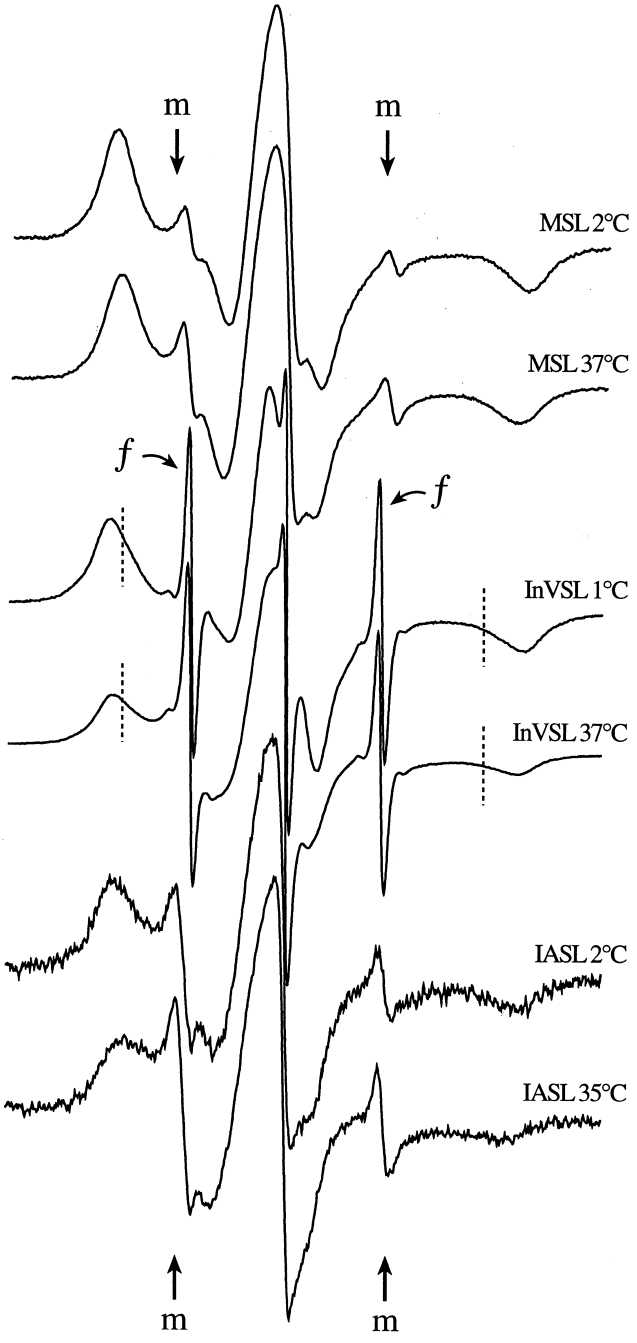

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Fig. 3. Conventional V_1 EPR spectra of GalP membranes spin labelled with 6-MSL (top pair), 5-InVSL (middle pair), or IASL (bottom pair). Spectra were recorded at the temperatures indicated. Arrows labelled 'm' indicate a small mobile component in the spectra of 6-MSL and IASL. The sharp component labelled 'f' is 5-InVSL spin label free in solution. The spectral shoulders indicated by dashed lines correspond to bound 5-InVSL in a state of intermediate immobilisation. For all three spin labels, the major species is strongly immobilised and gives rise to the outermost peaks with hyperfine splitting $2A_{max}$ that is defined in Fig. 2. Buffer: 50 mM potassium phosphate, 10 mM magnesium sulphate, pH 6.6. Total scan width = 10 mT.

more minor, spectral component (the shoulders indicated by dashed lines in Fig. 3) that displays somewhat higher mobility and a larger temperature dependence. This indicates some heterogeneity in the labelling of unprotected membranes with 5-InVSL.

The spectra of IASL in Fig. 3 indicate somewhat higher mobility than for the other two labels, for the following reasons. In addition to a small proportion of highly mobile groups (labelled 'm' in Fig. 3), the major part of the IASL spectrum does not approach so closely to the rigid limit. The outer hyperfine splittings of the major component are smaller and line widths of the outer peaks are larger for IASL than for the other two labels. This is indicative of a limited mobility in the slow motion regime of conventional nitroxide EPR spectroscopy (see, e.g., [17]), consistent with a rather more flexible link of the spin label to the point of covalent attachment that is characteristic of the iodoacetamide derivative.

Differences in mobility between the different labels can be seen more clearly from the temperature dependence. Fig. 4 shows the maximum outer hyperfine splittings, $2A_{max}$, of the three covalent labels as a function of increasing temperature. Not only are the values of $2A_{max}$ smaller for the iodoacetamide IASL spin label, but also the decrease in $2A_{max}$ with increasing temperature is much larger for this label. This clearly indicates a somewhat higher mobility for the IASL spin label that has the more flexible covalent attachment to the protein. The spectra of IASL still remain in the slow motion regime, however, at the higher temperatures (see Fig. 3). The value of $2A_{max}$ at 40°C is 6.2 mT, as compared with a value of approx. 3.4 mT for the very mobile spectral component. Small differences are seen between the 6-MSL and 5-InVSL spin labels in the values of $2A_{max}$. The temperature dependence for 6-MSL is slight, but that for 5-InVSL is even smaller, indicating a yet greater degree of immobilisation for the latter label. The main component of the spectra of 6-MSL in membranes without NEM prereaction has slightly smaller values of $2A_{max}$ than those from membranes prereacted with NEM, but a similar temperature dependence. The quantitative differences may point to spin labelling of a smaller proportion of groups with slightly higher mobility in the non-prereacted samples.

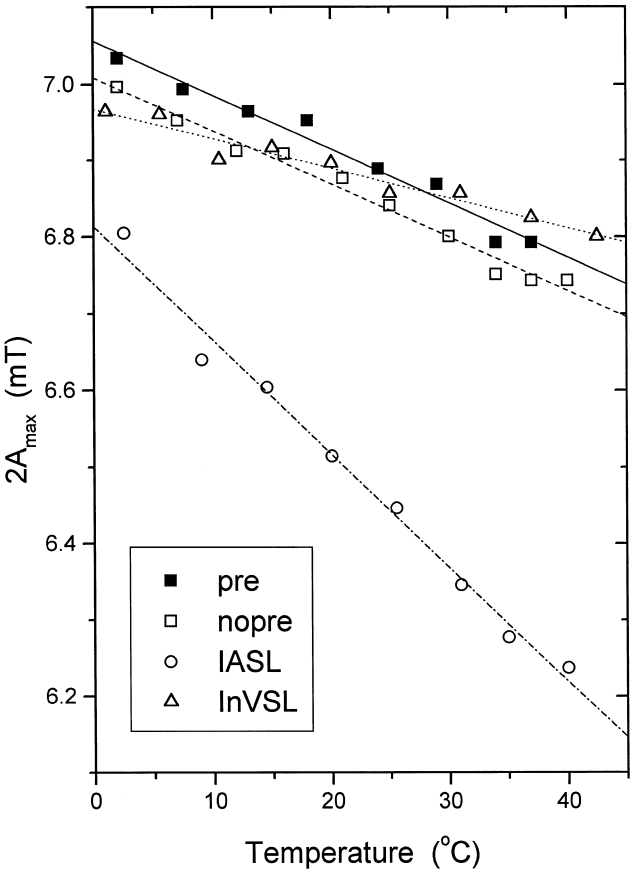

Fig. 4. Temperature dependence of the outer hyperfine splitting, $2A_{max}$, of the V_1 EPR spectra of GalP membranes spin labelled with 6-MSL (■, □), IASL (○), or 5-InVSL (△). Solid symbols are with, and open symbols without, prereaction with NEM in the presence of 20 mM D-galactose.

3.2. Saturation transfer EPR spectroscopy

The conventional V_1 EPR spectra of the specifically labelled GalP membranes demonstrate that there is very little independent segmental motion of the 6-MSL spin label relative to the whole protein (see Figs. 2 and 4). This affords a near-ideal situation in which to study the overall rotational motion of the entire protein assembly by saturation transfer EPR spectroscopy. The V_2' ST-EPR spectra of GalP membranes that were prereacted with NEM in the presence of 20 mM D-galactose and then post-labelled on the protected groups by 6-MSL, are shown in Fig. 5. Unlike the conventional V_1 EPR spectra, the V_2' ST-EPR spectra (which are sensitive to much slower motions) show a very pronounced change in line shape with increasing temperature (cf. Fig. 2). At

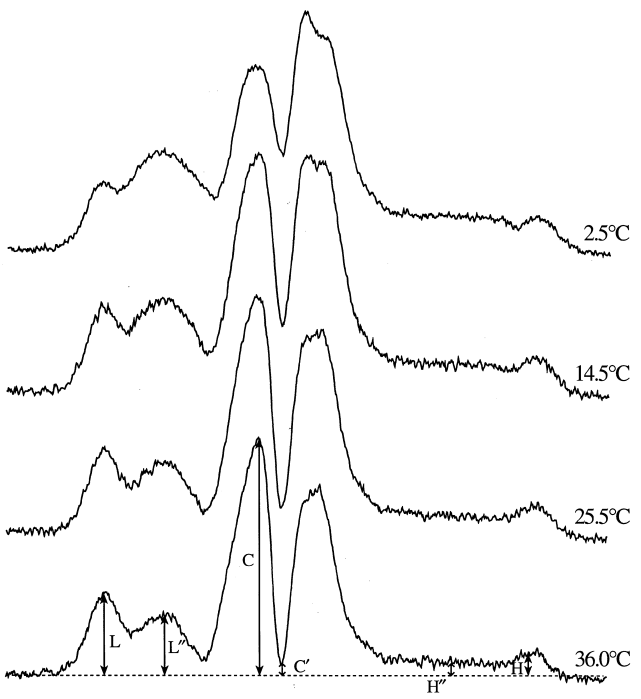

Fig. 5. Second harmonic, 90° out-of-phase, absorption ST-EPR spectra (V_2' display) of GalP membranes spin labelled with 6-MSL, after prereaction with NEM in the presence of 20 mM D-galactose. The temperatures at which the spectra were recorded are indicated in the figure. Buffer: 50 mM potassium phosphate, 10 mM magnesium sulphate, pH 6.6. Total scan width = 10 mT. For display, spectra are normalised to the same positive line height.

low temperatures (2.5°C), the spectra are indicative of rather low mobility on the microsecond timescale, with high relative intensities in the intermediate diagnostic regions at low, central and high field, and relatively high overall intensity (see e.g. [13,18]). With increasing temperature, the relative intensities in the diagnostic regions decrease, as does the overall intensity. This corresponds to a very marked increase in the overall rotational mobility of the protein on the microsecond time scale.

The normalised integrated intensity, I_{ST}, of the ST-EPR spectra in Fig. 5 is given as a function of temperature in Fig. 6 (solid squares). The normalised intensities drop rather rapidly with increasing temperature from 2.5°C, to a considerably lower value at 25–36°C. This is a clear demonstration of the increased rate of overall rotation of the protein at these higher temperatures. Returning to 3.5°C yields a value similar to that obtained initially at 2.5°C. Also shown in Fig. 6 are measurements from a sample that was not subjected to prereaction with NEM (open squares). In the main, the data are similar in magnitude and trend to those for the specifically labelled sample, except that an irreversible increase in intensity is observed on incubation at higher temperatures (37°C). On returning to low temperature (1°C), the intensity is higher than that predicted by extrapolation of the data obtained initially at low temperature. A similar phenomenon was observed previously with maleimide spin-labelled (Na+K)-ATPase [19] and is discussed in this latter reference.

The diagnostic line height ratios (L''/L, C'/C and H''/H in the low, central and high field regions, respectively [18]) and the overall integrated intensity (I_{ST}, [13]) of the ST-EPR spectra were used to determine effective rotational correlation times for the spin-labelled protein. This was done by comparison with standard calibration V_2' ST-EPR spectra of haemoglobin spin labelled with 6-MSL, in isotropic solutions of known viscosity [13]. The calibration parameters were taken from [14], and the results are given in Table 1. Because the spin-labelled integral protein rotates with axial anisotropy in the membrane, the rotational correlation times obtained from isotropic calibrations are only effective values and will depend on the orientation, θ, of the spin label principal z-axis to the axis of rotation, i.e. to the membrane normal [17,20]. This is seen directly from a comparison of the effective correlation times deduced from the central (C'/C), and low and high field (L''/L and H''/H) diagnostic line height ratios.

Table 1
Effective rotational correlation times deduced from diagnostic line height ratios (τ_L, τ_C, τ_H for low field, central and high field regions, respectively) and normalised integral intensity, τ_I, in the ST-EPR spectra of GalP membranes specifically spin labelled with 6-MSL after NEM prereaction in the presence of excess D-galactose[a]

T (°C)	τ_L (μs)	τ_C (μs)	τ_H (μs)	τ_I (μs)
2.5	240	24	150	260
3.5	150	12	130	240
14.5	81	9	98	53
25.5	44	6	61	28
36	34	5	56	27

[a]Deduced from calibrations of the diagnostic spectral parameters for haemoglobin spin labelled with 6-MSL in isotropic glycerol-water solutions of known viscosity [13,14].

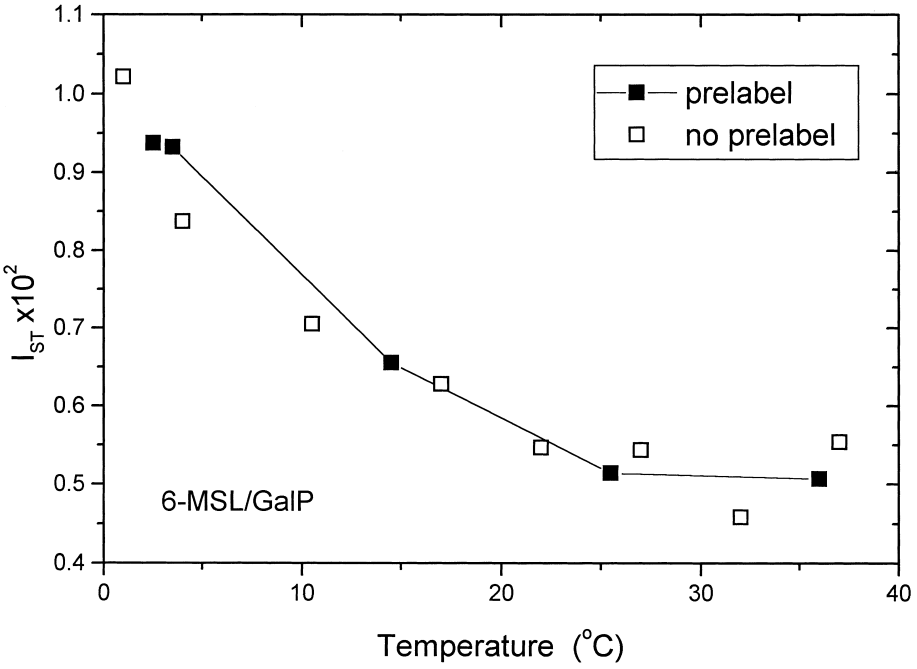

Fig. 6. Temperature dependence of the normalised integrated intensities, I_{ST}, of the V_2' ST-EPR spectra of GalP membranes spin labelled with 6-MSL after prereaction with NEM (■) and without prereaction with NEM (□) in the presence of 20 mM D-galactose.

Whereas the latter yield similarly long effective correlation times, those deduced from the central region of the spectrum are much shorter (Table 1). This is because the central line height ratio is sensitive only to rotation around the *z*-axis of the nitroxide [21,22]. On the other hand, the low and high field ratios are sensitive primarily to off-axis rotations about the membrane normal, if the nitroxide *z*-axis is preferentially parallel to the latter [20,22]. It will be noted that the central region of the ST-EPR spectrum is often obscured by overlapping spectral components from spin labels with higher mobility, and therefore cannot be used to determine the anisotropy of the overall protein rotation. In the present case, the spectrum of GalP membranes prereacted with NEM consists of a single immobilised spectral component, without any contamination from more mobile components, and therefore may be used reliably for this purpose (cf. [21]).

4. Discussion

4.1. Spin label binding site

The labelling site on GalP that is protected by excess D-galactose leads to complete inhibition of sugar transport when reacted with standard sulphydryl labelling reagents [1]. Mutagenesis studies, in which cysteine residues were replaced systematically by serine, have identified this protectable site of sulphydryl labelling as Cys^{374} (see Fig. 1), and NEM and eosin-maleimide labelling of cysteine replacement mutants has also demonstrated that Cys^{374} on GalP is the only major labelling site of inner membranes containing GalP [7]. Spin labelling without NEM prereaction in the presence of D-galactose gives rise to EPR spectra of 6-MSL that consist mainly of a component strongly resembling the single component obtained on specifically labelling the protected site (compare Figs. 2 and 3). This is consistent with Cys^{374} on GalP being the major site of spin labelling in inner membranes with overexpressed GalP. The results obtained with 5-InVSL (see Fig. 3, dashed lines) suggest, however, that some of the non-specific spin-labelled sites may have spectra

rather similar to those of spin-labelled Cys^{374} on GalP.

Selective spin labelling with 6-MSL at the unique protectable site yields EPR spectra that are characteristic of a homogeneous and highly constrained environment. Spin labelling with other sulphydryl reagents also reveals that the major site of labelling is highly constrained. Even for the iodoacetamide spin label, which can yield spectra indicative of a rather mobile environment, e.g. with the Ca-ATPase [23], the conventional EPR spectra indicate rather strong immobilisation. The environment of the critical sulphydryl group on GalP is therefore rather restricted in nature. This situation is not, of course, wholly unique. Generally, non-specific spin labelling of membrane-bound proteins with maleimide derivatives gives rise to spectral components from immobilised groups, as well as from mobile groups. However, the proportion of the latter is frequently greater than is found here with non-specific labelling of GalP membranes. Strong immobilisation might be a characteristic of transmembrane segments. More specifically though, strong immobilisation is a consequence of high local packing density within the protein. Comparison with a recent site-directed spin-labelling study on the single transmembrane segment of the M13 phage coat protein [24] reveals that an intramembranous environment per se is insufficient to generate the degree of immobilisation obtained with specifically labelled GalP. Only for a spin-labelled residue trapped within the interhelical hinge region of the bitopic M13 coat protein was the local packing density sufficiently high to immobilise the spin label to the extent found here for GalP (see also [25]).

Other studies have indicated that mutation of the neighbouring residue Trp^{371} strongly affects the affinity of GalP for sugar [26]. Together with the mutagenesis studies on Cys^{374}, the present spin-labelling results therefore are consistent with close proximity of these residues to a sugar binding site that is blocked sterically by sulphydryl labelling and can discriminate between D- and L-galactose in the unblocked state.

4.2. Overall protein rotational diffusion

The ST-EPR spectra of the 6-MSL spin label specifically attached at the GalP binding site, after prereaction with NEM in the presence of excess D-galactose, clearly indicate the anisotropy of rotation of the GalP protein in the membrane (Table 1). This only arises if the principal spin label z-axis has a fixed orientation, θ, relative to the membrane normal. Under these conditions, the correlation time, $\tau_{R//}$, for rotation of the whole protein around the membrane normal is related to the effective correlation times τ_L, τ_H obtained from the low and high field line height ratios by [20]:

$$\tau_{R//} = \tfrac{1}{2}\tau_{L,H} \sin^2 \theta \quad (1)$$

where any off-axis wobble of the protein is assumed to have an insignificant effect on the ST-EPR spectra. An upper limit for the rotational correlation time of the protein is therefore given by $\tau_{R//} = \frac{1}{2}\tau_{L,H}$, corresponding to $\theta = 90°$ in Eq. 1. From Table 1, it is clear that this corresponds to rather slow motion on the microsecond timescale, even at 36°C. A more direct estimate is given by the line height ratios determined from the central region of the ST-EPR spectrum, i.e., $\tau_{R//} \approx \tau_C$, because the latter is sensitive only to rotation around the nitroxide z-axis (i.e., to x-y axis interchange) [22]. Correlation times for overall protein rotation that are deduced from τ_L and τ_H using Eq. 1, are relatively consistent with these τ_C values when a fixed value of $\theta \approx 26°$ that does not vary appreciably with temperature is assumed for the spin label orientation (cf. Table 1).

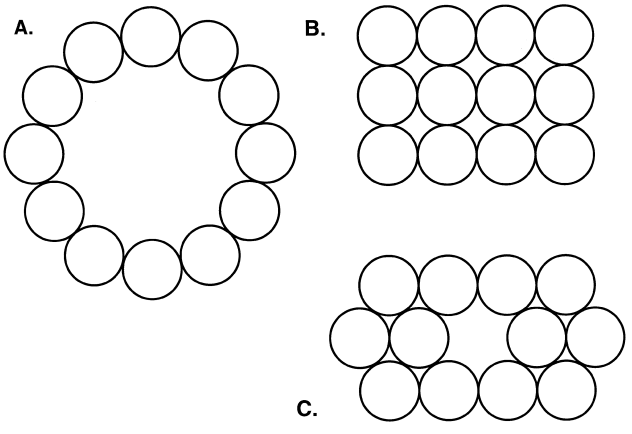

Fig. 7. Idealised symmetrical packing arrangements for 12 transmembrane α-helices. (A) Regular dodecagon with a large internal pore ($\tau_{R//} \approx 6.2$ μs). (B) Rectangle of square-packed helices ($\tau_{R//} \approx 3.5$ μs). (C) Close-packed trilayer helical sandwich with a single central vacancy ($\tau_{R//} \approx 4.5$ μs).

The rotational correlation time that is predicted for an integral membrane protein from hydrodynamic models is given by (see e.g. [17]):

$$\tau_{R//} = \pi \eta h(a^2 + b^2)/(3kT) \quad (2)$$

where η ($\approx$5 P [27]) is the membrane viscosity, h ($\approx$4.5 nm) is the height of the intramembranous section of the protein, and a and b are the semi-axes of its (assumed) elliptical cross-section. Other symbols have their usual meaning. Assuming an approximately circular cross-section, the effective diameter of the GalP protein deduced from the τ_C data given in Table 1 for 36°C is $2a \approx 4.4$ nm. For comparison, idealised symmetrical packing arrangements for the intramembranous section of monomeric GalP are shown schematically in Fig. 7. A regular polygonal arrangement of 12 transmembrane helices (of cross-sectional diameter 1.0 nm) would have an outer diameter of approx. 4.9 nm (and an inner pore diameter of approx. 2.9 nm). For this configuration, Eq. 2 predicts that $\tau_{R//} \approx 6.2$ μs. A square-packed arrangement of 12 transmembrane helices would have cross-sectional dimensions in the region of $a \approx 2.0$ nm, $b \approx 1.5$ nm, which corresponds to a rotational correlation time deduced from Eq. 2 of $\tau_{R//} \approx 3.5$ μs. A symmetric trilayer sandwich structure of 12 helices with a single central vacancy (pore diameter 1.0 nm), would have $a \approx 2.5$ nm, $b \approx 1.4$ nm, corresponding to a rotational correlation time of $\tau_{R//} \approx 4.5$ μs. From these comparisons, it is clear that the rotational correlation times deduced from τ_C at physiological temperatures are reasonably consistent with an assembly of 12 transmembrane helices that are less tightly arranged than is predicted for close packing of helices that are not tilted relative to the membrane normal. Uncertainties are associated with the value of 5 poise taken for the membrane viscosity, which corresponds to the upper bound determined from rotational diffusion studies of bacteriorhodopsin in a single component fluid bilayer membrane [27]. GalP constitutes approx. 50% of the total protein in the *E. coli* plasma membranes used. Protein-protein interactions might be expected to contribute to the effective viscosity in *E. coli* membranes. Certainly both protein-protein interactions and/or changes in the effective lipid viscosity must be invoked to explain the rather large decrease in rotational mobility of GalP at low temperatures (approx. 2°C).

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References

[1] P.J.F. Henderson, A.J.S. Mcpherson, Assay, genetics, proteins, and reconstitution of proton-linked galactose, arabinose, and xylose transport systems of *Escherichia coli*, Methods Enzymol. 125 (1986) 387–429.

[2] M.C.J. Maiden, M.C. Jones-Mortimer, P.J.F. Henderson, The cloning, DNA sequence, and overexpression of the gene *araE* coding for arabinose-proton symport in *Escherichia coli* K12, J. Biol. Chem. 263 (1988) 8003–8010.

[3] P.J.F. Henderson, The 12-transmembrane helix transporters, Curr. Opin. Cell Biol. 5 (1993) 708–721.

[4] S.A. Baldwin, P.J.F. Henderson, Homologies between sugar transporters from eukaryotes and prokaryotes, Annu. Rev. Physiol. 51 (1989) 459–471.

[5] P.E. Roberts, The Galactose-H^+ Symport Protein of *Escherichia coli*, Ph.D. Thesis, University of Cambridge, Cambridge, 1992.

[6] P.J.R. Spooner, N.G. Rutherford, A. Watts, P.J.F. Henderson, NMR observation of substrate in the binding site of an active sugar-H^+ symport protein in native membranes, Proc. Natl. Acad. Sci. USA 91 (1994) 3877–3881.

[7] T.P. McDonald, The Molecular Dissection of the Galactose-H^+ Symport Protein (GalP) of *Escherichia coli*, Ph.D. Thesis, University of Cambridge, Cambridge, 1994.

[8] M. Esmann, H.O. Hankovszky, K. Hideg, J.A. Pedersen, D. Marsh, Vinyl ketone reagents for covalent protein modification. Nitroxide derivatives suited to rotational diffusion studies by saturation transfer electron spin resonance, using membrane-bound Na,K-ATPase as an example, Anal. Biochem. 189 (1990) 274–282.

[9] M. Futai, Experimental systems for the study of active transport in bacteria, in: B.P. Rosen (Ed.), Bacterial Transport, Marcel Dekker, New York, 1978, pp. 7–41.

[10] A. Ward, N.M. Sanderson, J. O'Reilly, N.G. Rutherford, B. Poolman, P.J.F. Henderson, The amplified expression, identification, purification, assay and properties of histidine-tagged bacterial membrane transport proteins, in: S.A. Baldwin (Ed.), Membrane Transport – A Practical Approach, Blackwell Press, Oxford, 2000, pp. 131–166.

[11] P. Fajer, D. Marsh, Microwave and modulation field inhomogeneities and the effect of cavity Q in saturation transfer ESR spectra. Dependence on sample size, J. Magn. Reson. 49 (1982) 212–224.

[12] M.A. Hemminga, P.A. de Jager, D. Marsh, P. Fajer, Standard conditions for the measurement of saturation transfer ESR spectra, J. Magn. Reson. 59 (1984) 160–163.
[13] L.I. Horváth, D. Marsh, Analysis of multicomponent saturation transfer ESR spectra using the integral method: application to membrane systems, J. Magn. Reson. 54 (1983) 363–373.
[14] D. Marsh, Exchange and dipolar spin-spin interactions and rotational diffusion in saturation transfer EPR spectroscopy, Appl. Magn. Reson. 3 (1992) 53–65.
[15] M. Esmann, H.O. Hankovszky, K. Hideg, D. Marsh, A novel spin-label for study of membrane protein rotational diffusion using saturation transfer electron spin resonance. Application to selectively labelled class I and class II -SH groups of the shark rectal gland Na^+/K^+-ATPase, Biochim. Biophys. Acta 978 (1989) 209–215.
[16] L.I. Horváth, L. Dux, H.O. Hankovszky, K. Hideg, D. Marsh, Saturation transfer electron spin resonance of Ca^{2+}-ATPase covalently spin-labeled with β-substituted vinyl ketone- and maleimide-nitroxide derivatives. Effects of segmental motion and labeling levels, Biophys. J. 58 (1990) 231–241.
[17] D. Marsh, L.I. Horváth, Spin-label studies of the structure and dynamics of lipids and proteins in membranes, in: A.J. Hoff (Ed.), Advanced EPR. Applications in Biology and Biochemistry, Elsevier, Amsterdam, 1989, pp. 707–752.
[18] D.D. Thomas, L.R. Dalton, J.S. Hyde, Rotational diffusion studied by passage saturation transfer electron paramagnetic resonance, J. Chem. Phys. 65 (1976) 3006–3024.
[19] M. Esmann, L.I. Horváth, D. Marsh, Saturation-transfer electron spin resonance studies on the mobility of spin-labeled sodium and potassium ion activated adenosinetriphosphatase in membranes from *Squalus acanthias*, Biochemistry 26 (1987) 8675–8683.
[20] B.H. Robinson, L.R. Dalton, Anisotropic rotational diffusion studied by passage saturation transfer electron paramagnetic resonance, J. Chem. Phys. 72 (1980) 1312–1324.
[21] D. Marsh, Molecular motion in phospholipid bilayers in the gel phase: long axis rotation, Biochemistry 19 (1980) 1632–1637.
[22] P. Fajer, D. Marsh, Sensitivity of saturation transfer ESR spectra to anisotropic rotation. Application to membrane systems, J. Magn. Reson. 51 (1983) 446–459.
[23] C. Coan, G. Inesi, Ca^{2+}-dependent effect of ATP on spin-labeled sarcoplasmic reticulum, J. Biol. Chem. 252 (1977) 3044–3049.
[24] D. Stopar, K.A.J. Jansen, T. Páli, D. Marsh, M.A. Hemminga, Membrane location of spin-labeled M13 major coat protein mutants determined by paramagnetic relaxation agents, Biochemistry 36 (1997) 8261–8268.
[25] D. Bashtovyy, D. Marsh, M.A. Hemminga, T. Páli, Molecular modelling studies on the spin-labelled major coat protein of the M13 bacteriophage in a phospholipid bilayer, (2000) submitted for publication.
[26] T.P. McDonald, A.R. Walmsley, G.E.M. Martin, P.J.F. Henderson, The role of tryptophans 371 and 395 in the binding of antibiotics and the transport of sugars by the D-galactose-H^+ symport protein (GalP) from *Escherichia coli*, J. Biol. Chem. 270 (1995) 30359–30370.
[27] R.J. Cherry, R.E. Godfrey, Anisotropic rotation of bacteriorhodopsin in lipid membranes, Biophys. J. 36 (1981) 257–276.