

Rotational Mobility and Orientational Stability of a Transport Protein in Lipid Membranes

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ABSTRACT A single-cysteine mutant of the lactose transport protein LacS(C320A/W399C) from *Streptococcus thermophilus* was selectively labeled with a nitroxide spin label, and its mobility in lipid membranes was studied as a function of its concentration in the membrane by saturation-transfer electron spin resonance. Bovine rhodopsin was also selectively spin-labeled and studied to aid the interpretation of the measurements. Observations of spin-labeled proteins in macroscopically aligned bilayers indicated that the spin label tends to orient so as to reflect the transmembrane orientation of the protein. Rotational correlation times of 1–2 μ s for purified spin-labeled bovine rhodopsin in lipid membranes led to viscosities of 2.2 poise for bilayers of dimyristoylphosphatidylcholine (28°C) and 3.0 poise for the specific mixture of lipids used to reconstitute LacS (30°C). The rotational correlation time for LacS did not vary significantly over the range of low concentrations in lipid bilayers, where optimal activity was seen to decrease sharply and was determined to be $9 \pm 1 \mu$ s (mean \pm SD) for these samples. This mobility was interpreted as being too low for a monomer but could correspond to a dimer if the protein self-associates into an elongated configuration within the membrane. Rather than changing its oligomeric state, LacS appeared to become less ordered at the concentrations in aligned membranes exceeding 1:100 (w/w) with respect to the lipid.

INTRODUCTION

The lactose symport protein LacS of *Streptococcus thermophilus* belongs to the large class of secondary transport systems that comprises of several dozen families (Nikaido and Saier, 1992; Poolman and Konings, 1993). As with many of these transport proteins, LacS is predicted to fold into 12 transmembrane helices but is unusual in that it carries a large (19.5 kDa) hydrophilic C-terminal extension that serves a regulatory function (Poolman et al., 1989, 1995).

LacS has been overexpressed and purified from native membranes to investigate conditions required for optimal reconstitution of this and other transport systems into lipid membranes (Knol et al., 1996). Optimal reconstitution of detergent-solubilized LacS occurred in lipid bilayers that were destabilized with nonsolubilizing amounts of detergent (Knol et al., 1996, 1998). However, as illustrated in the current study, functionally competent reconstitution was only achieved at very low protein concentration; lipid/protein ratios (L/P) greater than 100–200 by weight, with specific activities reducing sharply to low levels as the protein concentration was increased to around L/P = 50 (w/w). A detailed account of these observations will be reported elsewhere. As with practically all other transport systems, the oligomeric state of LacS in the membrane is

unknown. The purpose of the current study was to determine the oligomeric state for LacS, functionally reconstituted at low protein concentration into a defined lipid membrane, and further to determine whether this varies as the specific activity decreases at the higher protein concentrations.

Mutants have been engineered to replace the native cysteine in LacS (C320A) and to substitute a single cysteine into the transmembrane helix XI or the interhelix loop 10–11, regions predicted to be close to the substrate binding site in the protein (Poolman et al., 1996; Spooner et al., 1999). Mutants not defective in transport are K373C (interhelix loop 10–11) and W399C (transmembrane helix XI). Despite its predicted location at the midpoint of helix XI, Cys³⁹⁹ was accessible to maleimide reagents and is conceivably located within a translocation channel in the protein. The LacS(C320A/W399C) mutant was therefore selected as the most likely one to provide an environment where the dynamics of the spin label will reflect solely the overall rotational motion of the protein.

The saturation transfer electron spin resonance (ST-ESR) method (Thomas et al., 1974) provides access to a motional time scale appropriate for observing the rotational mobility of proteins in membranes (10^{-6} to 10^{-3} s). The method usually involves an empirical interpretation of line shapes in terms of rates of isotropic motion, the relationship of which to the actual uniaxial mobility in membranes is not straightforward. The strict interpretation in terms of rotation about the membrane normal requires that the spin label adopt a unique orientation in the protein and that this orientation can be defined for the experimental system. Freed and co-workers have described how macroscopic alignment of systems for ESR (Ge et al., 1994), especially lipid membranes, can greatly improve spectral resolution and the ability to

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derive detailed information not readily accessible from normal dispersion samples. For the current study, conventional ESR observations were made of the reconstituted membranes that were macroscopically aligned to obtain information on the orientation of spin label in the protein. The results indicate that the short-chain spin label used can adopt a preferred orientation within the reconstituted protein, and this leads to a less ambiguous determination of rotational motion within the membrane. Knowledge of spin-label orientation also allows access to other physical parameters that are important for interpreting protein mobilities such as the effective membrane viscosity and could provide a sensitive measure of whether the protein adopts a regular orientation in its reconstituted state.

MATERIALS AND METHODS

LacS(C320A/W399C)

S. thermophilus ST11(Δ lacS) carrying plasmid pGKHis was grown semi-aerobically at 42°C in Elliker broth supplemented with 0.5% (w/v) beef extract, 20 mM lactose, and 5 μ g/ml erythromycin. Using a polymerase chain reaction approach (Knol et al., 1996), Trp³⁹⁹ was replaced with cysteine, using the lacS(C320A) gene in pGKH(C320A) as the parent. The oligonucleotide used to construct lacS(C320A/W399C) was 5'CA.AAC.TGT.CTT.GTG.TCG.ACA.TTT (mutations are underlined). In addition to the TGG (Trp) to TGT (Cys) change, a BstEII site was created 3 bp downstream from the TGT codon. The fragment containing the mutant sequence was checked by nucleotide sequencing, and the resulting plasmid was named pGKH(C320A/W399C).

All protein purification steps were performed at 4°C. The protein was purified essentially as described (Knol et al., 1996), with the following modifications. Membranes of *S. thermophilus* were isolated and solubilized in 15 mM imidazole (pH 8.0), 10% (v/v) glycerol, 100 mM NaCl, plus 0.5% (w/v) dodecyl maltoside (DDM). After 10–20 min of incubation, insoluble material was removed by centrifugation (280,000 \times g, 15 min). The solubilized membrane proteins were mixed and incubated for 30 min with Ni-NTA resin (Qiagen; ~4 mg of LacS/ml of resin) that had been equilibrated with buffer A (15 mM imidazole (pH 8.0), 10% (v/v) glycerol, 100 mM NaCl, plus 0.05% DDM). The column material was loaded into a Bio-Spin column (Bio-Rad Laboratories) and washed with 10 column volumes of buffer A containing 25 mM imidazole. The protein was eluted with 200 mM imidazole (pH 7.0) containing 0.05% DDM. Subsequently, the protein was diluted 10-fold with buffer B (5 mM HEPES (pH 7.0) containing 0.05% DDM). The diluted protein sample was applied to a Bio-Spin column containing Q-Sepharose fast flow resin (Pharmacia Biotech; 10 mg of LacS/ml of resin) that had been equilibrated with buffer B. After washing with 10 column volumes of buffer B plus 25 mM NaCl, the protein was eluted with buffer B containing 100 mM NaCl. Peak fractions usually contained 0.6–0.8 mg/ml of LacS. The LacS protein was concentrated using Microcon-100 filters (Amicon), which avoided concentrating the surfactant that was not associated with the protein. The LacS concentration was determined from the A_{280} using $\epsilon = 76,320 \text{ M}^{-1} \text{ cm}^{-1}$ (Pace et al., 1995) after the appropriate corrections for light scattering (Leach and Scheraga, 1960).

Spin labeling of LacS(C320A/W399C) was carried out in 200 mM imidazole (pH 7.0) containing 10% glycerol and 0.05% (w/v) DDM at a LacS concentration of 0.34 mg/ml (i.e., 4.8 μ M). Protein was incubated with a fivefold molar excess of 3-maleimido-proxyl spin-label (Fluka) for 1 h at room temperature. The reaction mixture was then diluted ninefold, and unreacted label was removed by column chromatography on Q-Sepharose, as described above.

For protein reconstitution, liposomes of acetone/ether-washed *Escherichia coli* lipids and egg yolk phosphatidylcholine (Avanti Polar Lipids) in the ratio 3:1 (w/w) were made by freeze-thawing a aqueous dispersion of the lipid mixture (20 mg/ml) in 50 mM phosphate buffer (pH 7.0), containing 2 mM MgSO₄, followed by extrusion through polycarbonate filters with a 400-nm pore size (Mayer et al., 1986). Extruded liposomes at 4 mg/ml of lipid were titrated with the DDM until saturated with surfactant, before the onset of solubilization (Knol et al., 1998), and then incubated (30 min at 20°C) with solubilized protein at the required ratios with gentle agitation. To remove the detergent, polystyrene beads (SM2 Biobeads, Biorad) were added at a wet weight of 80 mg/ml, and the sample was incubated for a further 2 h at room temperature. Fresh Biobeads were then added twice and equilibrated at 4°C with the samples, first for 3 h and then overnight. The proteoliposomes were collected by centrifugation, washed with 50 mM potassium phosphate (pH 7.0), and stored in liquid nitrogen or at –70°C. Samples prepared at the L/P used in this work showed no differences in gross morphology, according to freeze-fracture electron microscopy. Samples for analysis were thawed rapidly at 37°C and sedimented by ultracentrifugation (200,000 \times g, 10 min) or aligned on glass plates as described below for reconstituted rhodopsin.

Lactose counterflow activity was determined for proteoliposomes that been extruded through 400-nm polycarbonate filters after thawing. These samples were equilibrated in 50 mM phosphate buffer (pH 7.0) with 2 mM MgSO₄ and 10 mM lactose, for 2 h at room temperature. After the sample was concentrated by centrifugation, 1- μ l aliquots of the proteoliposome suspension were diluted into 200 μ l of the phosphate buffer, containing 2 mM MgSO₄ and 9 μ M [¹⁴C]lactose, making a final lactose concentration of 59 μ M. The assay was stopped at various time intervals by diluting the mixture with 2 ml of ice-cold 0.1 M LiCl, and the proteoliposomes were collected on 0.45- μ m cellulose nitrate filters. The filters were washed with 2 ml of 0.1 M LiCl, and the retention of radiolabeled lactose was measured by liquid scintillation counting.

Bovine rhodopsin

All preparative and analytical procedures involving bovine rhodopsin were carried out in dim red light. Rod outer segments were isolated from fresh bovine retinas (Papermaster, 1982), and these were washed with hypotonic buffer (2.5 mM Tris-HCl buffer at pH 7.4 with 1 mM MgCl₂) to release the disc membranes. The intact disc membranes containing the rhodopsin were collected by centrifugation (180,000 \times g, 20 min) and rinsed with 15 mM HEPES buffer (pH 7.0, containing 120 mM NaCl and 1 mM EDTA). Discs were resuspended in the HEPES buffer to give a rhodopsin concentration of ~2 mg ml⁻¹, according to the absorbance measured at 500 nm ($\epsilon = 40 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) for aliquots diluted into a 1% (w/v) solution of hexadecyltrimethylammonium bromide.

Spin labeling of rhodopsin in the disc membranes was carried out after more accessible cysteines were blocked with *N*-ethyl maleimide. A procedure similar to that described here (Kusumi et al., 1980) was shown to restrict the reaction with maleimide spin label to a single cysteine within the protein. Disc membranes were first pretreated with 1 mM NEM at 4°C for 1 h and centrifuged, and the membrane pellet was then rinsed in the HEPES buffer four times. The rhodopsin in the disc membranes was then incubated for 16 h at 4°C with a fourfold molar excess of the 3-maleimido-proxyl spin label (Sigma), rinsed twice with the HEPES buffer, and then solubilized in 50 mM Tris-HCl buffer (pH 7.0) containing 50 mM sodium acetate, 1 mM CaCl₂, 2 mM MnCl₂, using 1% (w/v) octylglucoside as the surfactant (Alexis Corp., San Diego, CA). The protein was purified by affinity chromatography (De Grip, 1982) on concanavalin A-Sepharose (Sigma). Once adsorbed onto a small column of concanavalin A-Sepharose (<5 ml), the material was exhaustively washed with the octylglucoside Tris-HCl buffer (at least 50 bed volumes) before the protein was eluted with the detergent buffer containing 200 mM methyl mannoside. The rinsing procedure described here was found to be crucial to ensuring that the purified protein was free from other spin-labeled membrane compo-

nents. The eluted protein solution was diluted fivefold and concentrated twice with detergent buffer alone, using PM30 ultrafiltration membranes, and then finally concentrated to ~ 1 mg/ml of protein for reconstitution.

The solubilized spin-labeled protein was mixed with either the *E. coli*-egg phosphatidylcholine (PC) reconstitution lipid mixture or dimyristoylphosphatidylcholine (DMPC), both of which had been solubilized by adding a minimal volume of sodium cholate solution (5% w/v) to their hydrated suspensions. The L/P used was 3.4 (w/w) for the mixed reconstitution lipids or 3.5 (w/w) for DMPC, both of which correspond to ~ 200 lipids per rhodopsin, based on an average molecular weight for the *E. coli* and egg PC lipid mixture of 700. The protein-lipid-surfactant mixtures were dialyzed at 4°C against 1 L of 5 mM HEPES reconstitution buffer (pH 7.5 with 50 mM NaCl, 0.5 mM EDTA, and 0.1 mM sodium azide) that was changed frequently over a period of 14 days, with the final stages of detergent removal assisted by the addition of SM2 Biobeads to the dialysis buffer. The reconstituted protein could then be sedimented by ultracentrifugation ($200,000 \times g$, 10 min) for loading into the capillary tubes used for the ESR measurements. Alternatively, samples of the proteins reconstituted into lipid membranes were aligned on glass plates (0.8×0.8 cm) by the method of isopotential spin-dry ultracentrifugation (Clarke et al., 1980; Gröbner et al., 1997). Typically, each plate was loaded with a thin film composed of 0.3 mg of lipid with up to ~ 2 nmol of protein in the case of rhodopsin. This level of loading typically gave films with a uniform appearance and alignment when examined at low magnification with a microscope with crossed polarizing filters. Occasionally, membrane films appeared to be nonuniform to the naked eye and these were not used in the analysis. Samples of aligned membranes were equilibrated at room temperature in a chamber at 92% relative humidity for 4 h, before four to eight of the plates were stacked together and wrapped in polytetrafluoroethylene tape to prevent drying.

ESR spectroscopy

Conventional and ST-ESR measurements were made with a Bruker ESP 300 spectrometer operating at 9 GHz (X-band). Dispersion (nonaligned) samples of membranes were loaded in capillary tubes as line samples, not exceeding 5 mm, and were maintained at 28°C or 30°C for the measurements. Quartz glassware was used in the standard TE₁₀₂ microwave cavity to optimize measuring sensitivity. Plates containing aligned membranes were secured in a quartz tube that replaced the standard Dewar assembly in the cavity. The aligned samples were consequently analyzed only at ambient temperature ($20 \pm 1^\circ\text{C}$) without instrumental temperature control. The plates were supported with their faces parallel to the sides of the tube, and the tube was rotated in the cavity to change the angle of the plates with respect to the direction of the magnetic field. Procedures used for the ST-ESR measurements broadly correspond to those described previously (Thomas et al., 1974). Microwave power levels were adjusted to 0.25G to record ST-ESR spectra, after calibration with a deoxygenated, 0.9 mM solution of peroxylamine disulfonate (Aldrich) in 10 mM potassium carbonate, with a sample configuration similar to that used for dispersion samples. Calibration of the isotropic rotational correlation times for this method was accomplished using spin-labeled hemoglobin solution as a standard for isotropic mobility. Hemoglobin (human, double recrystallized; Sigma) was spin-labeled as a solution of 100 mg/ml in 100 mM phosphate buffer at pH 6.8, with two equivalents of 3-maleimido-proxyl spin-label for 1 h. After extensive dialysis, the solution was centrifuged ($200,000 \times g$) to remove any aggregated protein and concentrated (~ 200 mg/ml) by ultrafiltration (Centricon, Amicon). The viscosity of the protein solution was adjusted by preparing mixtures with glycerol (Ultrapur; BRL Life Technologies, Gaithersburg, MD), according to standard viscosity tables (Sheeley, 1932), and using empirical temperature relationships (Slie et al., 1966) to provide an appropriate range of isotropic correlation times predicted from the Debye relation (Eq. 1). General details of the ST-ESR measurements were as described previously (Thomas et al., 1974).

Data treatment

The experimental strategy followed here was designed to incorporate all of the information required to properly interpret ST-ESR measurements in terms of the anisotropic rotational mobility of membrane-reconstituted LacS and then to estimate the effective size of this protein within the membranes. The first stage of the analysis involves the empirical treatment of experimental ST-ESR spectra by comparing their diagnostic peak height ratios, as described (Thomas et al., 1974), with those measured from standard samples of spin-labeled hemoglobin, the isotropic rotational correlation time of which is obtained from the Debye relation

$$\tau_R^{\text{iso}} = 4\pi\eta r^3/3kT \quad (1)$$

A comparison with this calibration of isotropic mobility yields an apparent rotational correlation time for the experimental system, τ_R^{app} , that takes no account of the particular anisotropic features of its motion. The theoretical treatment of ST-ESR line shapes under anisotropic motion (Robinson and Dalton, 1980) provides some basis for interpreting the apparent mobilities in terms of diffusion around (D_{\parallel} ; rotational) and perpendicular (D_{\perp} ; “wobbling”) to a single axis. As recognized previously (Esmann et al., 1987), for highly anisotropic rotation ($D_{\parallel} \gg D_{\perp}$) and within a range of correlation times of interest here, the apparent correlation time has the following components:

$$\tau_R^{\text{app}} = 1/\{3[D_{\parallel} \sin^2 \theta + D_{\perp}(1 + \cos^2 \theta)]\} \quad (2)$$

where θ is the angle between the nitroxide z axis and the axis of rotation. As illustrated in Fig. 1, in the molecular fixed principal axis system, the z

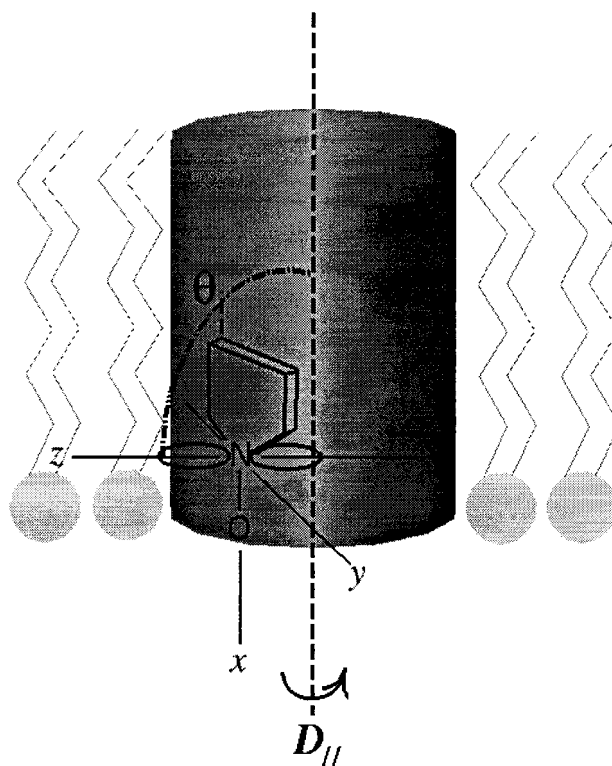


FIGURE 1 Schematic diagram of a spin label attached to a transmembrane segment of a membrane protein, showing the orientation of the nitroxide z axis in the pyrrolinyl ring, corresponding to the major component of the hyperfine interaction tensor, with respect to the axis around which the protein rotates in the membrane (at D_{\parallel}).

axis of the hyperfine interaction tensor is selected to lie along the $2p\pi$ orbital for the unpaired spin, perpendicular to both the plane of the pyrrolidyl ring and the x axis, located along the N—O bond. The apparent correlation time is most sensitive to the anisotropic rotation of the spin label when the z axis is oriented perpendicular to the rotation axis ($\theta = 90^\circ$) as shown (Fig. 1), whereupon Eq. 2 reduces to

$$\tau_R^{\text{app}} = 1/3D_{\parallel}$$

and because

$$D_{\parallel} = 1/6\tau_R \quad (3)$$

then the corresponding actual correlation time for this motion will assume its upper limit of

$$\tau_R = \tau_R^{\text{app}}/2 \quad (4)$$

As the nitroxide z axis tends toward the axis of rotation, the observed correlation time becomes progressively less sensitive to the anisotropic motion until it fails to detect the motion when these axes are coincident ($\theta = 0$).

Anisotropic (uniaxial) rotational diffusion can be related to the effective dimensions of the protein within the membrane by selecting a suitable hydrodynamic model, the simplest being that for anisotropic rotational diffusion of a cylinder with circular cross section about its long axis (Saffmann and Delbruck, 1975):

$$D_{\parallel} = kT/4\eta V_e \quad (5)$$

where V_e is the effective volume of the cylinder within the membrane. In the current work, bovine rhodopsin is used as a standard of known dimensions that can be selectively spin-labeled to estimate the viscosity of the mixed lipid bilayers used for reconstituting the LacS protein. Measurements are also obtained for bovine rhodopsin reconstituted into DMPC to compare with the previous measurement of viscosity for membranes of this lipid. However, as outlined above, the proper interpretation of anisotropic mobility requires some knowledge of the orientation of spin label within both of these proteins. An assessment of the orientational uniformity of the nitroxide in these proteins was therefore accomplished from observations of reconstituted protein that was macroscopically aligned on glass plates, with a view to determining the orientation (θ) of the major component of the **A** tensor (z axis) with respect to the diffusional axis in the membrane (see Fig. 1).

RESULTS

Specific activity of reconstituted LacS

Specific transport activity was determined from the initial rates of lactose counterflow in extruded vesicles with reconstituted LacS, and these measurements are shown in Fig. 2 as a function of the lipid-to-protein ratio. Despite the large excess of lipid present in these samples, optimal activity was not observed until L/P was ~ 400 (w/w). Specific transport activity dropped sharply when L/P was decreased below 200 (w/w) and leveled off at a low value around a L/P = 50 (w/w). Reconstitutions prepared using alternative surfactants (e.g., Triton X-100) show some differences in overall protein activity, but the dependencies on L/P were broadly very similar. More detailed activity measurements will be published elsewhere. The activity measurements for the reconstituted system studied here (Fig. 2) define a range of samples appropriate for the mobility measurements that

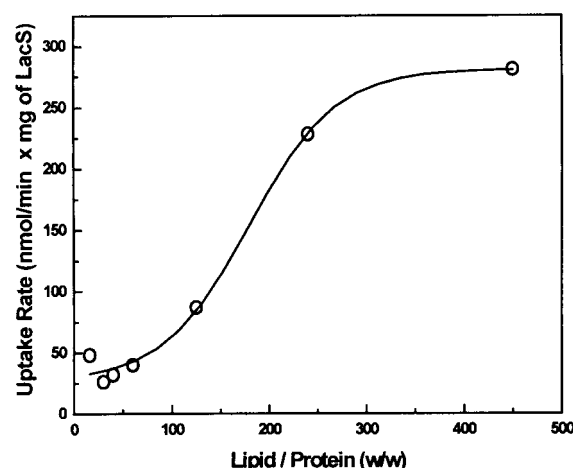


FIGURE 2 Dependence of LacS counterflow activity on the protein-to-lipid ratio in proteoliposome vesicles. Purified LacS was concentrated to 4 mg/ml and reconstituted at the L/P (w/w) as indicated. Specific conditions used for reconstitution are given in Materials and Methods. The transport activity was the initial rate of lactose counterflow.

is confined to extremely low protein concentrations (L/P ≈ 50 –400 by wt).

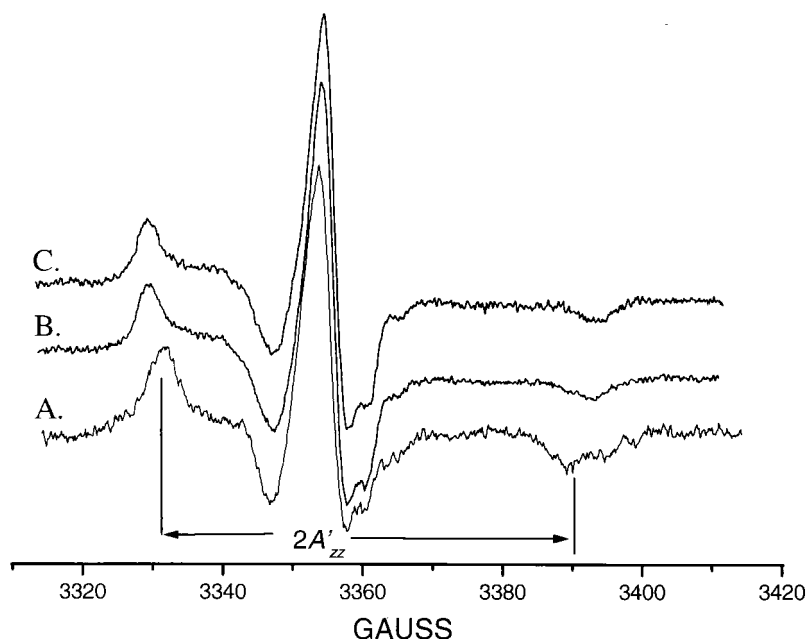
Conventional ESR on nonaligned samples

Conventional ESR spectra recorded from the nonaligned (dispersion) samples of reconstituted spin-labeled proteins are shown in Fig. 3, and each is indicative of a single population of motionally restricted nitroxide spin label. The outer spectral splitting, arising from the largest component of the nitroxide hyperfine interaction tensor (A'_{zz}), was close to 60 G for LacS (Fig. 3 A) in the *E. coli*-egg PC lipid mixture and 65 G for rhodopsin in either the lipid mixture or DMPC alone (Fig. 3, B and C). The splitting from nitroxide in rhodopsin can be assumed to represent the rigid limit value of A_{zz} and therefore displays no significant motion for this site in either reconstitution system on this time scale ($<10^{-8}$ s). The somewhat smaller splitting observed from nitroxide in reconstituted LacS protein suggests that the spin label in this case experiences some local mobility on this time scale. The motion represented here, however, is still highly constrained and is not assumed to interfere with the detection of rotational motion occurring over the longer time scales in the ST-ESR experiments. None of the spectra from the spin-labeled proteins showed any free or more mobile nitroxide components that can seriously interfere with the analysis of line shapes obtained with ST-ESR.

ST-ESR of reconstituted LacS

After ensuring the removal of nonspecifically attached or more mobile nitroxide components, the greatest challenge to obtaining reliable mobility measurements from ST-ESR

FIGURE 3 Conventional ESR spectra for (A) nitroxide-labeled LacS(C320A/W399C) reconstituted into *E. coli*-egg PC lipids at 30°C and nitroxide-labeled bovine rhodopsin reconstituted into (B) *E. coli*-egg PC lipids at 30°C and (C) DMPC at 28°C. The splitting defines an experimentally determined value for the major component of the hyperfine interaction tensor (A'_{zz}).



were the extremely low protein concentrations required to observe either functional LacS (L/P as low as 400 by weight) or the range of sharply decreasing specific activity (down to L/P \approx 50 by weight), as illustrated in Fig. 2. Second-harmonic, 90° out-of-phase ST-ESR spectra recorded from spin-labeled LacS(C320A/W399C), membrane reconstituted at a L/P between 26 and 400 (w/w), are shown in Fig. 4. Because of the low levels of spin-labeled protein, the absorption signal, especially from the weaker samples (higher L/P), show extraneous background interference in the high-field region of the spectra. In comparison, the signal in the low-field region is quite well defined and appears to be free from spectral distortion. Features in this low-field region exhibit high sensitivity to axial motion, over a wider range of correlation times, than do the strong features in the central region of the spectra (Fajer and Marsh, 1983), and the former are relied upon solely for the analysis reported here. The diagnostic low-field peak height ratio L'/L , used throughout, is obtained as illustrated in Fig. 5 A for the spectrum from a rhodopsin sample. The data are summarized in Table 1 for all concentrations of reconstituted LacS. These values for reconstituted LacS show no systematic or significant change over the entire range of L/P used.

The L'/L ratios from nitroxide-labeled hemoglobin in aqueous-glycerol mixtures provided a good linear calibration over logarithmic values of τ_R^{iso} , the predicted (Eq. 1) correlation time for isotropic rotation, between 6 and 100 μ s. The linear least-squares fitted expression of $L'/L = 2.541 + 0.4310 \log_{10} \tau_R^{\text{iso}}$ ($r > 0.999$) was used to calculate the apparent rotational correlation time (τ_R^{app} in Table 1) of $\sim 18 \mu$ s for LacS reconstituted into the lipid mixture.

ST-ESR of reconstituted bovine rhodopsin

High-quality ST-ESR spectra could be recorded from spin-labeled bovine rhodopsin that was reconstituted at L/P of 200 (mol/mol), as shown in Fig. 5, A and B. The L'/L ratios for the protein were quite similar in the two types of reconstitution lipid (Table 1) and fell below the linear calibration range used for interpreting the transporter mobility, described above. Because this was the first time that such rapid rotational motion had been recorded for rhodopsin by ST-ESR, considerable care was taken to obtain a reliable calibration for the low-field diagnostic peak height ratio in the region of short correlation times. Calibration data were obtained from the nitroxide-labeled hemoglobin in three different aqueous-glycerol mixtures measured over a limited temperature range (20–30°C). The resulting log-linear calibration of L'/L for shorter correlation times is shown in Fig. 5 C to describe a part of the calibration that approaches and passes through a minimum, as reported previously (Thomas et al., 1974). Small errors in the composition of these samples are probably the main source of scatter in the calibration data, but these define the region of higher rotational mobility well enough for reliable prediction of the short apparent correlation times for rhodopsin, as given in Table 1 (τ_R^{app}).

Conventional ESR on aligned samples

Conventional ESR measurements of samples of reconstituted protein, aligned on glass plates, as a function of the angle, ψ , between the direction of the magnetic field and the director or diffusional axis of protein (membrane normal) are shown in Fig. 6. The director or membrane normal is

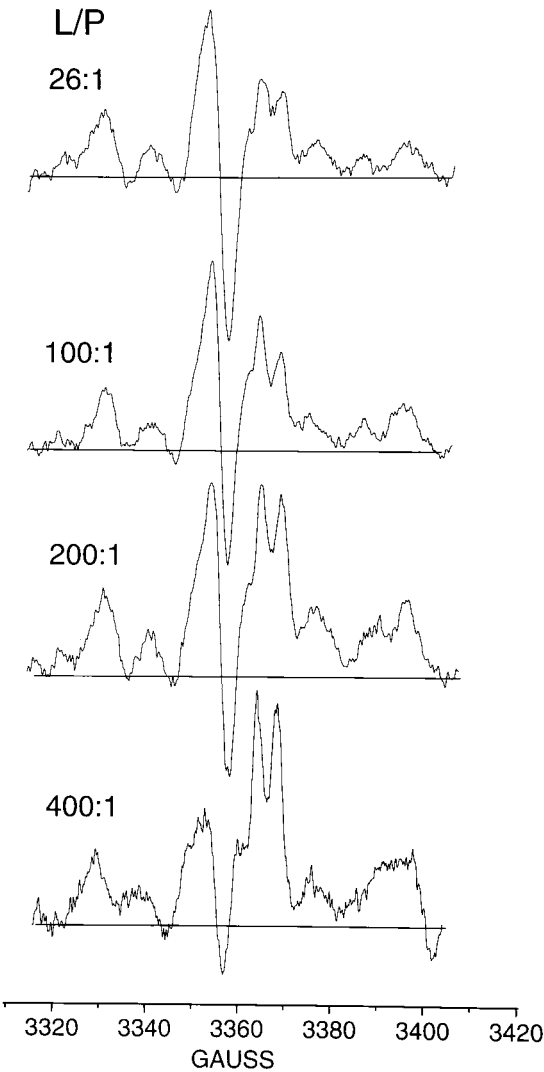


FIGURE 4 ST-ESR spectra recorded at 30°C from nitroxide-labeled LacS(C320A/W399C) reconstituted into the *E. coli*-egg PC lipid membranes at the L/P (w/w) given.

taken to be perpendicular to the face of the glass plates supporting the aligned membranes. The series of spectra in Fig. 6, A–C, show a strong dependence on the macroscopic sample orientation in the magnetic field. With a unique orientation of nitroxide in the protein, the *z* axis will form a single angle (θ) with the axis of ordering (director) that corresponds to the diffusion axis (see Fig. 1). The protein, however, is not ordered in the plane of the membranes, and so the *z* axis assumes all orientations around the director. Because the rotational mobility of the protein is very slow on the time scale of the conventional measurement, there is no significant averaging of this orientational distribution in the plane of the membranes, and so spectra do not simply display the characteristic strong features of the three hyperfine transitions. Instead, as shown in Fig. 6, A–C, the

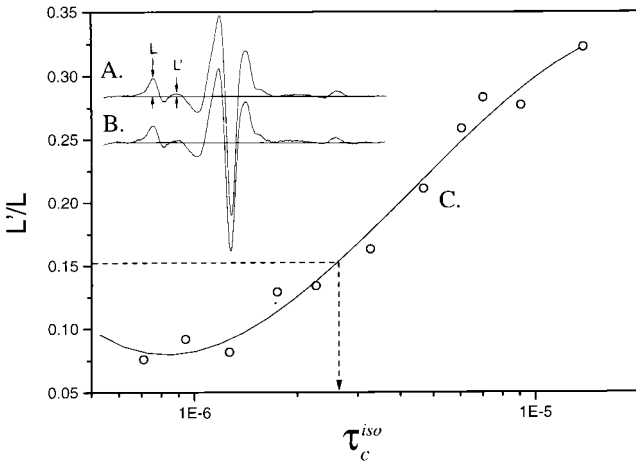


FIGURE 5 ST-ESR spectra from nitroxide-labeled bovine rhodopsin reconstituted into (inset A) *E. coli*-egg PC at 30°C or (inset B) DMPC at 28°C and (C) calibration plot for the low-field peak height ratio (L'/L) against the \log_{10} correlation time for isotropic rotation of nitroxide-labeled hemoglobin in aqueous glycerol mixtures with viscosities selected to define the short correlation time region.

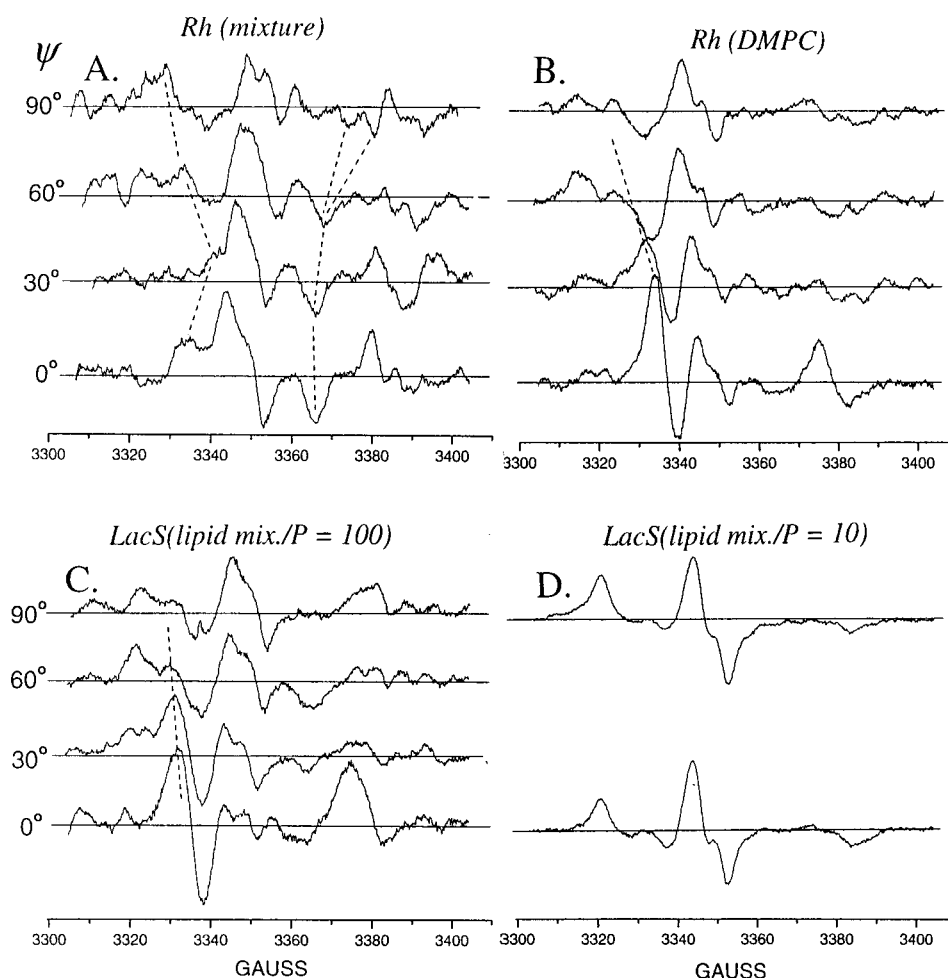
spectral anisotropy fragments into a complex arrangement of components, reflecting this nonaveraged orientational distribution in the *A* tensor. The advantage of the ultraslow protein rotation in these systems is that the conventional ESR line shapes can respond in a very sensitive way to a change in the orientation of the *A* tensor, especially the major *z* component, rather than to the orientation of the director axis. This should, in principle, be favorable for determining a unique orientation with respect to the director. The disadvantage of this situation is that the resulting line shapes are highly complex and difficult to resolve and properly interpret. In practice, it proved very difficult to obtain a satisfactory fit to the complex features in the spectra with a routine for simulating slow-motional ESR

TABLE 1 Rotational correlation times calculated from the ST-ESR measurements

Protein	Lipid	L/P (w/w)	L'/L^\dagger	τ_R^{app} (μs) [‡]	$\tau_{R\parallel}$ (μs) [§]
LacS	<i>E. coli</i> -egg PC	26:1	0.48	16	$9 \pm 1^\parallel$
	<i>E. coli</i> -egg PC	100:1	0.53	21	
	<i>E. coli</i> -egg PC	200:1	0.49	17	
	<i>E. coli</i> -egg PC	400:1	0.51	19	
Bovine rhodopsin	<i>E. coli</i> -egg PC	3.5:1*	0.151	2.6	1.7
Bovine rhodopsin	DMPC	3.4:1*	0.146	2.4	1.2

*Equivalent to molar ratios of lipid to rhodopsin of 200.
[†]Low field diagnostic peak height ratios from ST-ESR spectra (see Fig. 5, inset).
[‡]Values of apparent rotational correlation times.
[§]Uniaxial rotational correlation times around the bilayer normal, assuming $\theta = 90^\circ$ for LacS and bovine rhodopsin in DMPC and 60° for rhodopsin in *E. coli*-egg PC lipids.
^{||}Mean \pm SD for the range of LacS concentration.

FIGURE 6 Conventional ESR spectra recorded at 20°C from aligned membranes at various orientations, ψ , between the membrane normal and the direction of the membrane field. Aligned reconstituted membranes of nitroxide-labeled bovine rhodopsin were reconstituted into (A) *E. coli*-egg PC lipids at L/P = 3.5 (w/w) (L/P \approx 200 mol/mol) or (B) DMPC at L/P = 3.4 (w/w) (L/P = 200 mol/mol) and nitroxide-labeled LacS(C320A/W399C) was reconstituted into *E. coli*-egg PC lipids at (C) L/P = 100 (w/w) or (D) L/P = 10 (w/w).



line shapes (EPRL simulation program; Schneider and Freed, 1989). Recent work, combining observations at much higher fields (Barnes and Freed, 1998), has also emphasized the difficulties of analyzing spectral line shapes from aligned membrane samples recorded at the standard X-band frequencies. Instead, we rely on a more empirical interpretation of the gross changes observed in the spectra that show a distinct broadening and collapse in the spectral anisotropy with changes in sample orientation in the magnetic field, particularly in the low-field regions. These changes are traced by the broken lines in Fig. 6, A–C, which are not manifested as smoothly shifting splittings but rather as a redistribution of spectral intensity between outer and inner components, because of the lack of significant motional averaging in this case. Such behavior should only be possible for orientations of the nitroxide z axis that are close to being colinear with or orthogonal to the director, because it is only in these cases that the random distribution of orientations within the plane of the membrane can be effectively coaligned with or away from the direction of the magnetic field. Because the collapse in spectral anisotropy in the low-field region occurs with ψ between 30° and 0° for

rhodopsin in the *E. coli*-egg PC lipid mixture and at $\sim 0^\circ$ for both rhodopsin in DMPC and LacS in the *E. coli*-egg PC lipid mixture, then based on the above arguments, the z axis of spin label in each of these systems can be assumed to be directed away from the field and close to being orthogonal to the director ($\theta = 90^\circ$). Consistent with an orthogonal orientation of the z axes is the observation that these would appear to lie mostly along the magnetic field, giving maximum spectral anisotropy, when the director is positioned away from the field ($\psi = 90^\circ$).

The intensity changes occurring in the low-field regions of Fig. 6, A–C, appear to be largely complete, with little nonshifted signal, as far as can be determined at these measuring sensitivities. This provides a reasonable assurance that spin label does indeed tend to be homogeneously oriented with respect to the director axis and that the membranes are well aligned with respect to this axis. There is a suggestion in Fig. 6 C, however, that a small but significant portion of the spin label in LacS reconstituted at a L/P of 100 (w/w) may not be uniformly oriented and remains unshifted in the low-field region at $\psi = 0^\circ$. This is poorly defined at L/P = 100, but the proportion of nonshifted

signal increases progressively as the protein concentration is increased further, until at a L/P of 10 (w/w) the aligned membranes only display features characteristic of dispersion samples and show no significant change in line shape between $\psi = 0^\circ$ and 90° , as shown in Fig. 6 D. This would appear to represent a complete loss of uniformity in spin-label orientation at the higher protein concentration. The membrane films prepared at L/P = 10 (w/w) appeared to be as uniform and well ordered as those prepared at higher L/P, indicating that the protein itself was not well ordered in the membranes, at least in the environment of the attached spin label. Because of the lack of any homogeneous alignment at L/P = 10, no ST-ESR data were sought for this or any higher protein concentration in the lipid membranes.

DISCUSSION

The results obtained with bovine rhodopsin standard are not typical of the mobilities reported from ST-ESR for this protein and so warrant some discussion before being used for interpreting the measurements of membrane-reconstituted LacS. The specific site of spin labeling in rhodopsin was not determined here but should correspond to a cysteine that is located within the transmembrane portion of the protein and is most accessible to the cytoplasmic surface of the protein. According to recent structural predictions on the transmembrane portion of bovine rhodopsin (Pogozheva et al., 1997), these requirements would be best satisfied by Cys²²², whereas other possible candidates within the transmembrane segment, Cys¹⁶⁷ and Cys²⁶⁴, appear to be more deeply buried and closer to the intradiscal surface, based on this model (Pogozheva et al., 1997). In another spin-label study on rhodopsin (Farahbakhsh et al., 1992), Cys²²² was also identified as a likely site available for selective spin labeling, after cytoplasmic surface cysteines are blocked.

The apparent correlation times (τ_R^{app}) previously recorded from ST-ESR measurements of spin-labeled rhodopsin, based on the analysis in the low-field spectral region, have been quite variable but typically fall around 20 μs (Baroin et al., 1977; Kusumi et al., 1978). These mobilities were very different from that derived from recording the transient dichroism of rhodopsin in the frog photoreceptor membranes (Cone, 1972), which equates to a rotational correlation time of just 3.3 μs . We report here the first ST-ESR measurements of rhodopsin that are consistent with these optical measurements and are feasible for the size of this protein in its monomeric state. The actual rotational correlation times measured here (Table 1), between 1 and 2 μs , could be expected to be somewhat shorter than that determined in the photoreceptor membranes because of the higher lipid content in our reconstituted systems. These are equivalent to a L/P mol ratio of 200, as opposed to ~65–70:1 in the photoreceptor membranes (Daemen, 1973). Previous measurements (Kusumi et al., 1980) have indicated that the rotational mobility of rhodopsin reconstituted into

lipid membranes begins to decrease significantly at L/P below 150 (mol/mol), possibly because of some self-association of the protein. This can account for the nominal differences between the correlation times reported here for rhodopsin mobility and those measured optically in photoreceptor membranes, but not for the much longer correlation times obtained previously by ST-ESR. Insufficient purification of spin-labeled rhodopsin for the current study showed that trace amounts of more mobile spin-label impurity have a profound effect on the interpretation of ST-ESR spectra. Signal from these components adds to the portion of the spectra between the low-field turning points (around L' in Fig. 5 A), leading to anomalously high values of L'/L and consequently larger values of τ_R^{iso} . It was concluded that reliable use of ST-ESR for determining protein mobilities in membranes cannot tolerate even low levels of such impurity, and that previously reported measurements, predicting significantly lower mobilities than reported here, are likely to have been affected by small amounts of more mobile spin-labeled components.

The rhodopsin mobilities reported here can be applied with some confidence to characterize the lipid membranes used to reconstitute LacS. The crystallographic data currently available for bovine rhodopsin (Krebs et al., 1998) are good enough to allow the use of a hydrodynamic model that takes account of the nonspherical cross section that the protein presents to the membrane. The rotational diffusion of a protein with a symmetrical ellipsoidal cross section is given by (Jähnig, 1986)

$$D_{\parallel} = \frac{kT}{4\eta abh} \cdot \frac{2(a/b)}{[1 + (a/b)^2]} \quad (6)$$

where a and b are the semiaxes of the ellipsoid ($a > b$), and h is the membrane thickness, taken to be 45 Å. Using the values of $a = 18.4$ Å and $b = 12.4$ Å, taken from the current projection map of bovine rhodopsin in the plane of the membrane (Krebs et al., 1998), together with the rotational mobility data reported here, we obtain values for the lipid membrane viscosity of 3.0 poise for the *E. coli*-egg PC lipid mixture at 30°C and 2.2 poise for DMPC at 28°C. This is just below the range of 3.7 ± 1.3 poise predicted for DMPC at 28°C from transient dichroism measurement of the rotational mobility of bacteriorhodopsin (Cherry and Godfrey, 1981) and subsequently refined upward to 4.5 ± 1.5 poise by accounting for the noncircular cross section of this protein (Dornmair et al., 1985). In this entirely fluid lipid membrane, the mobility of bacteriorhodopsin showed no significant protein concentration effects between a molar L/P of 136 and 212 (Cherry and Godfrey, 1981), despite an earlier suggestion that these protein concentrations could exert large volume fraction effects on the lipid membrane (Cherry et al., 1978). The small (twofold) difference in mobility measured here for rhodopsin and that determined at the L/P = 65–70 (mol/mol) in photoreceptor membranes

indicates that such volume fraction effects are also not a significant factor in the current data obtained at L/P = 200 (mol/mol). The ST-ESR measurements of Kusumi et al. (1980) also show little or no concentration effects for rhodopsin in DMPC at L/P below 153 (mol/mol).

The greatest degree of uncertainty in the measurements made here lies in the estimation of orientations of the spin-label z axis. Arriving at somewhat lower viscosities for DMPC bilayers than reported by Cherry and Godfrey (1981) provides some confidence in the large or limiting values of θ deduced from the spectra of aligned membranes (60° in the *E. coli*-egg PC lipids and 90° in DMPC). This is because smaller orientations than used here would infer shorter correlation times for rhodopsin and even lower estimates of the membrane viscosity. Quite apart from the absolute values of membrane viscosity, it is generally preferred, wherever possible, to interpret results using those parameters obtained with the same technique. This is finally possible for interpreting the mobility of membrane reconstituted LacS, as described below.

The general conclusion from the observations of selectively labeled protein in aligned membranes, that spin label prefers to orient with its nitroxide z axes orthogonal to the membrane normal, corresponds to the case illustrated in Fig. 1 and is the orientation that exhibits maximum sensitivity to the rotational motion. The main constraining feature for this alignment may well be that the pyrrolidiny ring is made to coalign with the diffusion axis, because of favorable van der Waals interactions with surfaces of the transmembrane helical segments in the protein. The N-O bond, on the other hand, may be allowed all possible orientations within the x - y plane of the principal axis system, shown in Fig. 1. With $\theta = 90^\circ$ for the nitroxide z axis in spin-labeled LacS, the uniaxial rotational correlation time ($\tau_{R||}$) for LacS in the *E. coli*-egg PC lipid membranes is calculated to be $9 \pm 1 \mu\text{s}$ (mean \pm SD), by combining data over the entire range of protein concentrations studied by ST-ESR (Table 1). Using the simplest hydrodynamic model (Eq. 5) with the lipid viscosity determined here, this correlation time predicts an effective volume, V_e , for LacS of $1.9 \times 10^5 \text{ \AA}^3$ in the lipid membrane, which corresponds to an effective molecular mass of 150 kDa, using a partial specific volume of $0.76 \text{ cm}^3/\text{g}$. This is reasonably close to being twice the monomeric molecular mass of LacS, which is 69.5 kDa, but 19 kDa of this resides in a C-terminal extension or hydrophilic IIA domain that is not predicted to enter the bilayer.

The above calculation was based on the assumption of a circular cross section for LacS in the membranes, which will overestimate the effective volume of protein if the mobility of the protein is actually hindered by its presenting a flattened cross section to the membrane. A compact symmetrical ellipsoidal arrangement of the 12 transmembrane helices predicted for LacS is illustrated in Fig. 7; it has semiaxes $a = 23.5 \text{ \AA}$ and $b = 16.5 \text{ \AA}$, assuming an interhelical distance of 10 \AA . The appropriate hydrodynamic

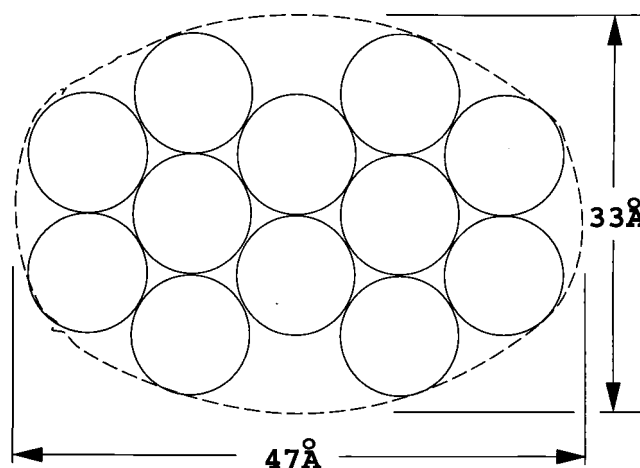


FIGURE 7 Idealized arrangement of 12 transmembrane helices into a symmetrical ellipsoidal configuration with axial dimensions based on a 10-\AA interhelix distance.

model (Eq. 6) predicts a rotational correlation time of $2.8 \mu\text{s}$ for a monomer having this configuration in the lipid membrane of viscosity determined here. This greater than three-fold difference from the observed mobility is expected to be well outside the experimental uncertainty in the methods. Judging by the mobilities measured for rhodopsin and the resulting values for membrane viscosity, it is also unlikely that the methods have greatly overestimated the correlation times for protein rotation in the membranes. A dimer of the idealized structure in Fig. 7 would have a correlation time of $5.1 \mu\text{s}$ if self-association extends along the shorter axes (side by side, giving $a = 31.0 \text{ \AA}$ and $b = 23.5 \text{ \AA}$) or $8.4 \mu\text{s}$ if dimerization were to extend along the longer axes (end to end, for $a = 47.0 \text{ \AA}$ and $b = 16.5 \text{ \AA}$). The experimentally determined correlation time for LacS in the lipid bilayers therefore fits quite well with a dimer, if we were to assume the most elongated configuration possible within the membrane. At the same time, we cannot rule out the possibility that the oligomeric state of LacS is larger than that of a dimer. In contrast to the LacS protein of *S. thermophilus*, the lactose transport system LacY of *E. coli* was deduced to be monomeric in DMPC membranes based on phosphorescence anisotropy measurements (Dornmair et al., 1985). This effective size was arrived at by the use of higher values of lipid membrane viscosity than determined here. It is therefore more relevant to compare the rotational mobilities alone for the two transport systems. The rotational relaxation time (inverse rotational diffusion coefficient) reported for LacY was $24 \mu\text{s}$ (Dornmair et al., 1985), which is equivalent to a correlation time of $4 \mu\text{s}$ (from Eq. 3), close to half that reported here for LacS. The differences in rotational correlation times measured between LacS and LacY could therefore reflect real differences in the oligomeric states of these proteins. Although the 12 helix transport proteins are often thought to have a monomeric struc-

ture in the membrane (Poolman and Konings, 1993), this study, as well as work currently undertaken on the oligomeric state of LacS in the detergent-solubilized state (Friesen and Poolman, unpublished work), strongly indicates that this is not generally true.

The mobility of LacS in the lipid bilayers did not vary significantly over the range of L/P between 400 and 26 (w/w), where the protein specific activity is seen to decrease sharply. Membranes that were macroscopically aligned on glass plates showed increasing amounts of randomly oriented nitroxide spin label as the protein concentration was increased above a L/P of 100 (w/w), until at a L/P of 10 (w/w) it was not possible to distinguish any remaining nitroxide in the protein that was uniformly oriented. In the absence of any other perturbation, the protein would be expected to orient well throughout this concentration range; the 10:1 weight ratio of lipid to protein still represents a 1000-fold molar excess of lipid, which is expected to support well the protein in the aligned membranes. It seems more likely, therefore, that these observations are detecting structural heterogeneity that develops in the protein at the higher concentrations in the lipid bilayers. These changes correlate roughly with the functioning of transporter because the activity (Fig. 2) and affinity constant (unpublished work) for transport were decreased sharply at a L/P around 100 (w/w). Interestingly, the reduction in activity of LacY with decreasing L/P, occurring at much higher concentrations in lipid membranes than studied here for LacS, has been attributed to a progressive increase in the average helical tilt of the protein in the lipid membranes (Le Coutre et al., 1997). The observations reported here for aligned membranes are difficult to account for in terms of a cooperative tilting of transmembrane segments of LacS in the membranes, unless this leads to a strong perturbation in the local structure around spin label in the protein.

A loss in uniform orientation of spin label was not evident in the ST-ESR measurements of the dispersion samples at higher protein concentration. This should not be taken to mean that such changes do not occur in the normal dispersion samples, but can be explained by the bias of the ST-ESR measurement toward the larger orientations of the nitroxide z axes with respect to the axis of rotational diffusion (membrane normal). The population distribution of the random orientations will also be skewed toward the larger orientations with respect to the single rotation axis. Conversion from uniform orientations, predicted here to be close to their limiting value (90°), to a random distribution of orientations will therefore retain sensitivity to the large orientations such that this change should prove difficult to detect by ST-ESR at the measuring sensitivities obtainable for the LacS system. Although the significance of these structural changes for LacS remains unclear, the use of aligned membranes for spatial observations of reconstituted protein containing judiciously located spin labels could

provide a valuable means of detecting structural perturbations that affect protein function.

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