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The size of the lactose permease derived from rotational diffusion measurements

Klaus Dornmair, Alan F.Corin[1], J.Keith Wright and Fritz Jähnig

Max-Planck-Institut für Biologie, Corrensstrasse 38, 7400 Tübingen and [1]Max-Planck-Institut für Biophysikalische Chemie, Am Fassberg, 3400 Göttingen, FRG

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The lactose permease of *Escherichia coli* was labeled with eosinyl-maleimide, reconstituted into vesicles of dimyristoylphosphatidylcholine and subjected to time-dependent phosphorescence anisotropy measurements in order to determine the rotational diffusion coefficient. By comparison with bacteriorhodopsin, the diffusion coefficient is evaluated in terms of an effective radius of the lactose permease in the plane of the membrane. This radius amounts to 20 ± 2 Å which implies that the lactose permease is a monomer. The monomeric state is maintained in the presence of a membrane potential.
Key words: lactose permease/rotational diffusion

Introduction

In the preceding paper, the folding of the lactose permease across the membrane has been studied. The present paper deals with the size of the protein. Centrifugation studies revealed that the permease solubilized in micelles of dodecylmaltoside is a monomer (Wright *et al.*, 1983b). The permease in membranes was shown by electron microscopy not to form large oligomers, however, monomeric and dimeric structures could not be distinguished from one another (Costello *et al.*, 1984). Finally, radiation inactivation studies indicated that the permease is a monomer in the absence of a membrane potential, but a dimer in the presence of a potential (Goldkorn *et al.*, 1984). This could imply that facilitated diffusion is mediated by the monomer, while active transport requires a dimer. However, instances are known where the technique of radiation inactivation provides misleading results (Jørgensen, 1982).

We therefore decided to investigate the state of aggregation of the lactose permease in the absence and presence of a membrane potential by performing rotational diffusion measurements. This technique has been shown to be an effective tool for studying the aggregation of membrane proteins (for a review, see Cherry, 1979). If applied to a well-defined system, it may even permit the determination of the radius of the protein. The point is that from the rotational diffusion coefficient either the protein radius can be derived if the viscosity of the membrane is known, or the viscosity can be derived if the protein radius is known. The latter has been performed by Cherry and Godfrey (1981) for the system bacteriorhodopsin in dimyristoylphosphatidylcholine (DMPC). Therefore, we reconstituted the lactose permease in the same lipid in order to determine the radius of the permease, in the absence and presence of a membrane potential. The rotational diffusion was detected via the time-dependent phosphorescence of the eosin-labeled protein (Austin *et al.*, 1979).

Results and Discussion

Rotational diffusion coefficient of the lactose permease

The time-dependent phosphorescence anisotropy of the eosin-labeled lactose permease in vesicles of DMPC is shown in Figure 1A for T = 28°C and $\Delta\psi = 0$. The fit by a mono-exponential decay with a finite residual anisotropy r_∞ is included and the residuals are also shown. The large time window of 2 ms was chosen to demonstrate the constancy of the residual anisotropy. Given the size of the vesicles, this implies that the anisotropy decay is not affected by vesicle rotation and solely reflects the rotational diffusion of the protein. For quantitative evaluation of the data, the time window was diminished to 200 μs. Figure 1B and C show the anisotropy in this time range for the same system at 28°C in the absence and presence of a membrane potential. The results of the fits for the relaxation times and amplitudes are listed in Table I. Because the results varied slightly between different samples, several samples were prepared, measured, and analyzed, and the mean values obtained are presented in Table I together with the error limits.

For five out of six samples the experimental data for the anisotropy could be well fitted by a mono-exponential decay and a residual anisotropy. Analysis for two relaxation times did not reduce the residuals of the fit. Only one out of six samples exhibited a double-exponential decay of the anisotropy (Table I).

As a test for the consistency of the experimental data with the theory for rotational diffusion of membrane proteins, the relaxation amplitudes $b_{1,2}$ and r_∞ were evaluated for the angle θ between the phosphorescence dipole moment and the membrane normal using equation 4 (see Materials and methods). The result is included in Table I. In the case of mono-exponential anisotropy decay, the measured amplitude can, *a priori*, be identified with the amplitude of either the fast or the slow relaxation term, i.e., with b_1 or b_2 of equation 4. Identification with b_1 leads to $\theta = 55.5°$ and identification with b_2 to $\theta = 21.4°$, which closely agrees with $\theta(r_\infty) = 20.9°$. Therefore, the measured amplitude is assigned to the amplitude b_2, so that according to equation 5a (see Materials and methods) the measured relaxation time is $\phi_2 = D_\parallel{}^{-1}$. Using the value $\theta = 21°$, the amplitude of the fast relaxation term, equation 4a, results as $b_1 = 0.001$, which is too small to be experimentally observed. Hence, the experimental data are fully consistent with the theory of rotational diffusion. Only in the case of the sample exhibiting double-exponential anisotropy decay, the amplitudes $b_{1,2}$ and r_∞ lead to values of θ, which are not compatible with each other. These data, therefore, cannot be interpreted in terms of rotational diffusion alone.

The result for the rotational relaxation time ϕ_2 of the lactose permease in vesicles of DMPC at T = 28°C and $\Delta\psi = 0$ is $\phi_2 = 25.0 \pm 6$ μs. This leads to the rotational diffusion coefficient $D_\parallel = 4.0 \times 10^4$/s. In the presence of a membrane potential of at least 110 mV, we obtained $\phi_2 = 18.7 \pm 5$ μs, equivalent to $D_\parallel = 5.3 \times 10^4$/s. These values imply that the lactose permease does not change its state of aggregation in response to a membrane potential. The transition from a monomer to a

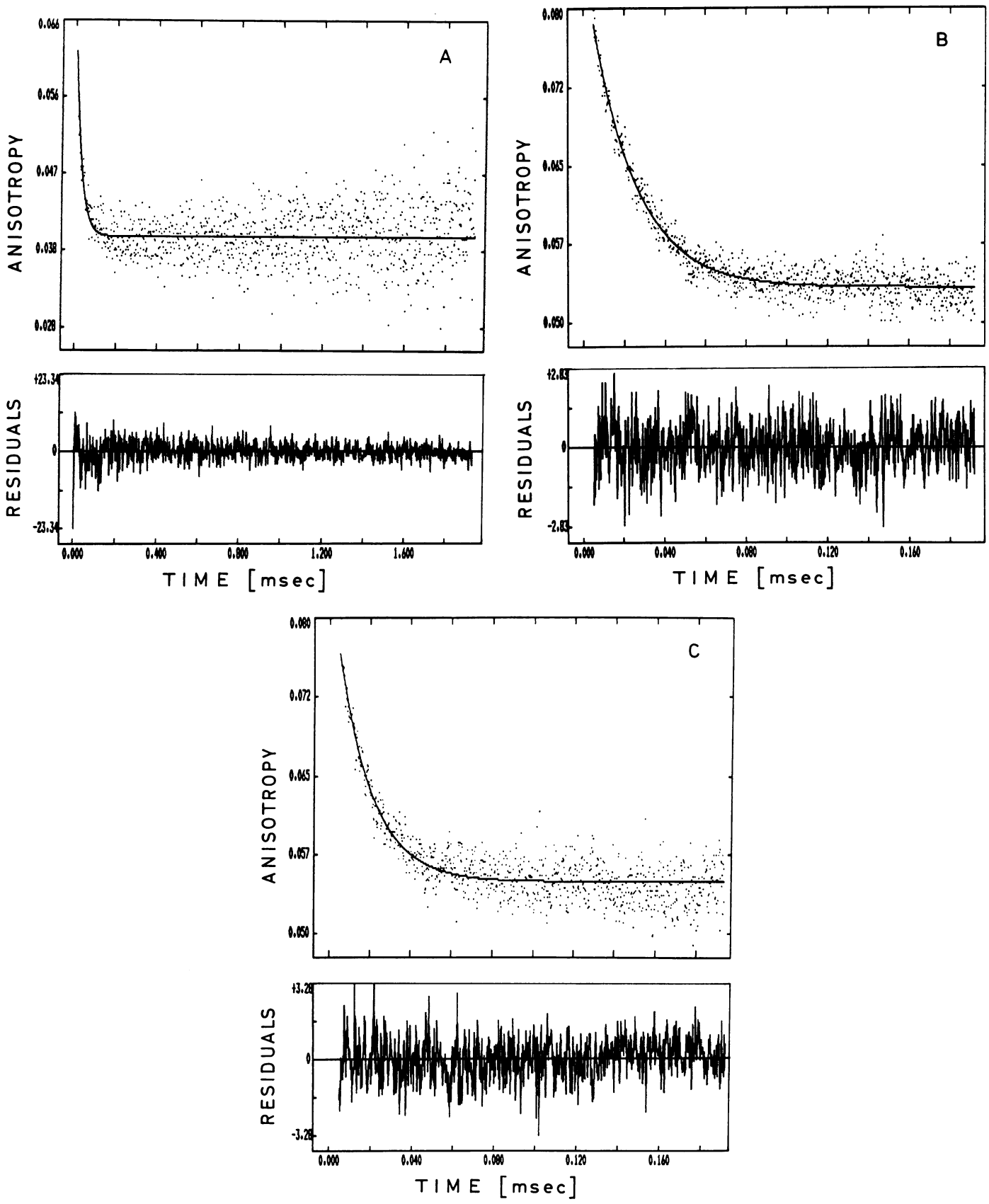

Fig. 1. Time-dependent phosphorescence anisotropy of eosin-labeled lactose permease in vesicles of DMPC at 28°C, for a time range of 2 ms and a membrane potential $\Delta\psi = 0$ **(A)**, and for a time range of 200 μs and $\Delta\psi = 0$ **(B)** or $\Delta\psi > 110$ mV **(C)**. The experimental data (dots), the fit by a mono-exponential decays plus a limiting anisotropy r_∞ (−), and the corresponding residuals (lower parts) are shown.

Table I. Rotational relaxation times and amplitudes of eosin-labeled lactose permease in vesicles of DMPC at T = 28°C in the absence and presence of a membrane potential $\Delta\psi$

	$\Delta\psi$ [mV]	N_s	N_m	ϕ_1 [μs]	b_1	ϕ_2 [μs]	b_2	r_∞	$\theta(b_1)$	$\theta(b_2)$	$\theta(r_\infty)$
S−R	0	5	12	–	–	25.0 ± 6	0.029 ± 0.005	0.055 ± 0.005	–	21.4	20.9
S−R	>110	3	4	–	–	18.7 ± 5	0.025 ± 0.005	0.058 ± 0.004	–	19.7	19.3
S−R	0	1	4	6.3	0.021	38.6	0.019	0.044	49.4	16.6	25.4
S−R	>110	1	3	3.2	0.028	36.1	0.019	0.045	52.9	15.8	26.6
R	0	1	2	–	–	26.3	0.039	0.039	–	27.4	26.2

The data for samples (S) were analyzed after subtraction of the data for reference samples (R). The results are presented as averages over the given number of measurements (N_m) performed on the given number of samples (N_s). The angles $\theta(b_1)$, $\theta(b_2)$ and $\theta(r_\infty)$ were calculated using equations 4a, b and c, respectively.

dimer as proposed by Goldkorn *et al.* (1984) would require the relaxation time to double according to equation 6 (see Materials and methods). Actually, however, it decreases slightly.

The size of the lactose permease

The state of aggregation of the lactose permease can be assessed by a quantitative evaluation of the diffusion coefficient. In the simplest approximation, one may assume a cylindrical shape for the permease and employ equation 6 to calculate the effective volume V_e. The value of the rotational viscosity $\eta_\parallel$ was derived from rotational diffusion measurements on bacteriorhodopsin in the same lipid DMPC at 28°C which yielded $\eta_\parallel = 4.5$ poise (see Materials and methods). With this value and $D_\parallel = 4 \times 10^4$/s one obtains $V_e = 58\,300$ Å^3 which corresponds to an effective mol. wt. of 47 200, using a partial specific volume of 0.74 cm^3/g. Like bacteriorhodopsin, the lactose permease probably consists of a number of membrane-spanning α-helices with short connecting loops (Vogel *et al.*, accompanying paper), hence the effective volume for rotational diffusion should closely agree with the actual volume and the effective mol. wt. with the actual mol. wt. Because the mol. wt. of the polypeptide chain is 46 500 (Büchel *et al.*, 1980), one must conclude from the rotational diffusion measurements that the permease is a monomer. This is also true in the presence of a membrane potential.

The approximate size of the lactose permease in the membrane plane can be determined if the thickness of the membrane equal to the height h of the cylinder describing the protein is known. Since the lipid used for the reconstitution is the same as in the case of bacteriorhodopsin, the same value for the thickness of the membrane should be used, $h = 45$ Å (Cherry and Godfrey, 1981). The effective volume $V_e = 58\,300$ Å^3 can then be evaluated for the effective cylinder radius a using equation 7 (see Materials and methods) and one obtains $a = 20 \pm 2$ Å.

One might even try to deduce information on the shape of the lactose permease from the diffusion coefficient. For this purpose, the assumed cross-section of the permease is generalized from a circle to an ellipse with half axes a_1 and a_2, and the further assumption is made that the effective volume V_e equals the actual volume $V = 57\,500$ Å^3 derived from the mol. wt. This assumption was introduced for bacteriorhodopsin (see Materials and methods) and, due to the probable similarities of the structures of bacteriorhodopsin and the lactose permease mentioned above, may also apply for the lactose permease. Using equation 6, the diffusion coefficient $D_\parallel$ can then be evaluated for the shape factor ν. The result is $\nu = 0.98$ which, according to equation 8 corresponds to an axial ratio $a_1/a_2 = 1.2$. With the value $F = V/h = 1280$ Å^2 for the area of the ellipse, one finally obtains $a_1 = 22 \pm 6$ Å and $a_2 = 18.5 \pm 4$ Å. The error limits here are larger than for a, because the dependence of $D_\parallel$ or ν on the axial ratio is weak. Hence, the determination of the shape of the lactose permease is not very reliable, the result simply indicates that the lactose permease is not extremely elongated.

In the model developed by Vogel *et al.* (accompanying paper), the lactose permease is postulated to consist of 10 membrane-spanning α-helices which are hydrophobic or amphipathic and form an outer ring and possibly four further helices which are more hydrophilic and fill the interior of the ring. This would yield a total of 14 membrane-spanning α-helices. Figure 2 demonstrates that an ellipse with half axes 22 Å and 18.5 Å as derived above, can, indeed, accommodate 14 α-helices. If 10 helices are located on the outer ring, the inner area is just sufficient for four helices. Hence, the rotational diffusion measurements support the above model for the lactose permease. The size of a lactose molecule is also indicated in Figure 2. It is unclear at present how this relatively large molecule gains access to its binding site and ultimately transverses the membrane through the protein.

Miscellaneous results

Three further results obtained from the rotational diffusion measurements are worth mentioning.

(i) The value of the relaxation time ϕ_2 was independent of whether the reference sample was subtracted from the sample or not. This indicates that the eosin groups of the reference samples are also located on the lactose permease. Indeed, when the phosphorescence anisotropy of the reference samples was analyzed, the same relaxation time as for the samples was obtained, only the amplitudes differed (Table I). Hence, the eosin groups of the reference samples are located at sites different from those of the samples and are oriented at a different angle relative to the membrane normal. The lactose permease thus seems to have at least one other group reactive with MalNEos.

(ii) Rotational diffusion measurements were also performed at temperatures above and below the lipid phase transition which for DMPC occurs at $T_t = 24$°C. The result for $D_\parallel$ is shown in Figure 3. As expected, the diffusion coefficient decreases upon passing through the phase transition. But the decrease is not as pronounced as in the case of bacteriorhodopsin (Cherry and Godfrey, 1981). The values of $D_\parallel$ at 28°C and 20°C differ only by a factor of ~2 for the lactose permease, but by an order of magnitude for bacteriorhodopsin. The slow rotation of bacteriorhodopsin at temperatures below T_t has been attributed to the known tendency of bacteriorhodopsin to aggregate and form a two-dimensional lattice. The result for the lactose permease might then indicate a reduced tendency for aggregation in the membrane plane.

(iii) The initial anisotropy of eosin attached to the lactose permease is $r_o = 0.084 \pm 0.010$ (Table I). This value is low

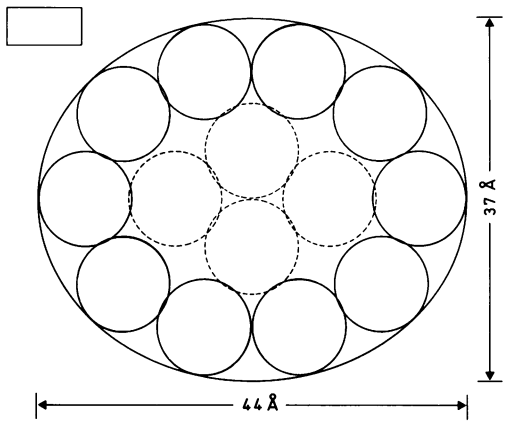

Fig. 2. Model for the packing of the lactose permease in the membrane plane. Solid circles represent 10 membrane-spanning α-helices predicted on the basis of their hydrophobicity, dashed circles four further membrane-spanning helices which are not predicted as membrane-spanning helices with certainty. The radius of an α-helix is assumed to be 5 Å. The rectangle illustrates the approximate size of a lactose molecule of dimensions 4 Å × 8 Å × 12 Å.

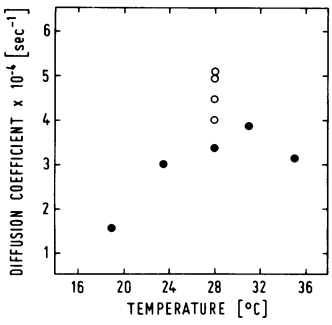

Fig. 3. Temperature dependence of the rotational diffusion coefficient of the lactose permease in vesicles of DMPC at $\Delta\psi = 0$. The results for a single sample (●), and for several samples at T = 28°C (○) are shown.

compared with the value of 0.4, expected theoretically for parallel absorption and emission moments and observed experimentally for eosin upon detection of fast fluorescence (Cross and Fleming, 1984). Such a difference is usually interpreted in terms of motions of the fluorophores in the nanosecond range (Kinosita *et al.*, 1984; Restall *et al.*, 1984; Mühlebach and Cherry, 1985). In our case, these motions may arise from motions of the eosin groups relative to the polypeptide backbone or from motions of the polypeptide backbone to which the eosins are attached, in this case the region around Cys 148. Such motions of the polypeptide backbone would be of special interest because of their possible correlation with conformational changes undergone by the permease upon sugar transport. They have been investigated by time-resolved fluorescence anisotropy measurements on the pyrene-labeled lactose permease which will be communicated in a forthcoming paper.

Materials and methods

Chemicals

N-(5-eosinyl) maleimide (MalNEos) was purchased from Molecular Probes (Junction City, OR), N-ethylmaleimide (MalNEt) from Serva (Heidelberg, FRG), DMPC from Fluka (Neu-Ulm, FRG), dodecyl-0-β-D-maltoside (DodOMalt) from Calbiochem (Giessen, FRG), and dodecyl/tetradecyl polyoxyethylene (Lubrol PX) from Sigma (München, FRG). Tetra-[^{3}H]phenylphosphonium bromide (^{3}H-Ph_4P Br) and [1-^{14}C]lactose were from Amersham (Braunschweig, FRG), and [6-^{3}H]lactose from CEA (Gif-sur-Yvette, France).

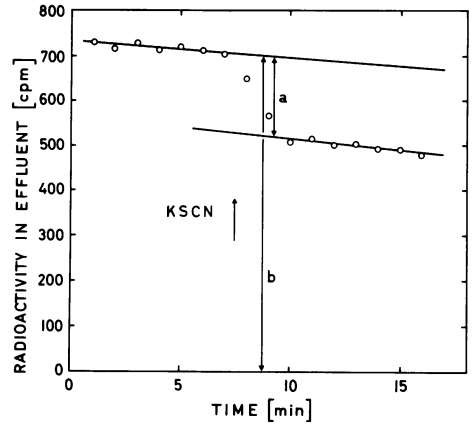

Fig. 4. Estimation of the membrane potential $\Delta\psi$ by flow dialysis. $\Delta\psi$ is imposed by making the vesicle suspension 0.2 M in KSCN and calculated according to equation 1.

Labeling and purification of the lactose permease

Membrane vesicles were prepared from cytoplasmic membranes of the overproducer strain *E. coli* T206 and pre-treated membranes obtained by blocking all reactive thiol groups except Cys 148 (Wright *et al.*, 1983a). Cys 148 was labeled with MalNEos in the same way as described for N-(1-pyrenyl)maleimide by Mitaku *et al.* (1984) except for the following modifications. 34 μmol MalNEos were dissolved in 10 ml methanol and sonicated for 10 min with 1 g diatomaceous earth. For each preparation 14 mg MalNEos-diatomaceous earth/ml suspension was used. The reaction was stopped by diluting the samples 2.5-fold with 1 mM cysteine in 50 mM phosphate buffer, pH 6.3. All reaction steps were carried out in the dark.

Control samples were prepared by incubating pre-treated membranes with 20 μl/ml of 0.1 M MalNEt (freshly prepared) for 60 min at 28°C before addition of MalNEos in order to inhibit labeling of Cys 148 by MalNEos. Further treatment of the control samples was identical to that of the samples, except that the reaction with MalNEos was stopped with 6 mM cysteine.

Purification of the lactose permease was performed by a slight modification of the method of Wright and Overath (1984). Instead of washing the membrane vesicles twice in 250 mM sodium 5'-sulfo-salicylate, they were suspended in 100 mM sodium 5'-sulfo-salicylate, 4 mM Na EDTA, 8 mM Tris, pH 7.5 to a protein concentration of 7.5 mg/ml, and 50 μl/ml 20% Na cholate was added (cf. Newman *et al.*, 1981). The suspension was sonicated for 10 min, diluted 5-fold with water, and centrifuged for 90 min at 150 000 *g* and 4°C. The samples were washed four times with water. For some of the samples, solubilization in DodOMalt was performed using 5 mM phosphate buffer, 20 mg/ml glycerol, pH 6.0 instead of 10 mM Tris-HCl, 1 mM EDTA, 20 mg/ml glycerol, pH 7.5. Because the reactive SH groups are labeled with MalNEt or MalNEos, addition of dithiothreitol to the buffers is not necessary.

Reconstitution of the lactose permease in DMPC

The labeled and purified lactose permease was reconstituted into DMPC vesicles in essentially the same way as described by Wright *et al.* (1983a) for reconstitution in *E. coli* lipids. The purified permease (typically 15 nmol) was diluted to 25 ml with the same buffer as used for Ecteola chromatography and the solution made 10 mM in Na EDTA and 120 mg/ml in glycerol. A lipid-detergent film on a glass vessel was prepared by dissolving 26 mg DMPC, 19.4 mg DodOMalt and 9.7 mg Lubrol PX in 3 ml benzene and evaporating the solvent at a temperature above 30°C. The lipid-detergent film and the protein solution were kept at 28°C for 20 min prior to addition of the protein solution to the film and sonication to clarity with an inserted glass rod. The important point is that the mixing is carried out at a temperature above the lipid phase transition temperature T_t which is 24°C for DMPC. To the clear solution 30 g (wet weight) polystyrene beads were added, and the detergent was removed by shaking the mixture slowly at 4°C for 18 h. The beads were removed by filtration. The vesicle suspension was washed once with a small amount of water, diluted 2-fold with water, and centrifuged for 90 min at 150 000 *g* and 4°C. The pellets were washed twice in 5 or 50 mM phosphate buffer, pH 7.5, suspended in 1.5 ml of the same buffer and stored as a stock suspension.

The eosin-labeled permease showed a single band in SDS polyacrylamide gel electrophoresis with an apparent mol. wt. of 31 000, the same value found with unlabeled permease. Also the elution profiles in Ecteola chromatography of labeled and unlabeled permease are identical. The yield of the purification and reconstitution was 30%, the same as for reconstitution in *E. coli* lipids (Wright and Overath, 1984). Substrate binding and transport could only be investigated with unlabeled

permease, because labeled permease does not bind substrate. 95% of all permease molecules reconstituted in DMPC exhibited normal binding and galactoside countertransport was detected at 30°C, i.e., above the lipid phase transition. According to Chen and Wilson (1984), the transport rate in DMPC is only ~10% of that in *E. coli* lipids. However, Raman measurements reveal that the secondary structure of lactose permease in DMPC and *E. coli* lipids is the same (Vogel *et al.*, accompanying paper). On the basis of these observations, we assume that eosin-labeled permease in DMPC has the same conformation as unlabeled permease in *E. coli* lipids.

Imposition of a membrane potential

A membrane potential was produced by addition of KSCN to the vesicle suspension. The magnitude of the potential was measured by flow dialysis using the same chamber as for substrate binding measurements (Wright *et al.*, 1983a). 0.5 ml of the reconstituted sample in 5 mM phosphate buffer, pH 7.5 was filled into the upper compartment, 10 μl of 2.8 μM [^{3}H]Ph_4P Br (2.5 MBq/ml) were added, and the suspension stirred gently for 10 min. The lower compartment (20 μl) was flushed with buffer, and seven fractions of 1.1 ml each were collected. After addition of 50 μl 2M KSCN another 10 fractions were collected. 1 ml of each fraction was diluted with 5 ml Quickscint 212 and the radioactivity determined in a liquid scintillation counter.

Figure 4 shows the radioactivity in the effluent plotted against time. Before addition of KSCN, the membrane-permeable cation [^{3}H]Ph_4P^+ is distributed equally between the internal and external volumes of the vesicles. When SCN^- enters the vesicles, and K^+ remains outside, [^{3}H]Ph_4P^+ follows the negative charge, and the radioactivity in the outer volume decreases. The membrane potential $\Delta\psi$ can be calculated from the Nernst equation:

$$\Delta\psi\ [\mathrm{mV}] = 59.9 \log \left(\frac{V_o}{V_i} \cdot \frac{a/b}{1 - a/b} + 1 \right) \quad (1)$$

with V_o and V_i denoting the external and internal volumes of the vesicles, respectively, and a and b are specified in Figure 4. For the ratio a/b we obtained 0.25 ± 0.04.

The inner volume V_i was determined by inclusion of [^{3}H]lactose in lipid vesicles. Vesicles suspensions of different concentrations of DMPC (5 – 15 mg/ml) in 5 mM phosphate buffer, pH 7.5 with 8.3 μM [^{3}H]lactose (0.37 MBq/ml) were prepared by rotary evaporation and sonication. Examination of the vesicles by negative staining electron microscopy showed that they were of the same size as those obtained by the reconstitution procedure. The vesicles were subjected to three freeze-thaw cycles, and 7.5 μl/ml of 26 μM [^{14}C]lactose (7.4 MBq/ml) were added. After centrifugation for 90 min at 150 000 *g* and 4°C, the pellet was resuspended in 0.5 ml water, 5 ml Quickscint 2000 was added, and the ^{3}H and ^{14}C radioactivities were measured using the supernatants as control for the concentrations of [^{3}H] and [^{14}C]lactose. Because [^{3}H]lactose is distributed homogeneously in the internal and external volumes, it appears both in the pellet and the supernatant, whereas [^{14}C]lactose remains in the supernatant. The difference in the concentrations of [^{3}H] and [^{14}C]lactose in the pellet, therefore, permits V_i to be determined. As a maximal value we found V_i = 0.70 μl/mg DMPC. Using this value and the minimal value a/b = 0.21, one obtains as a lower limit for the membrane potential $\Delta\psi$ = 112.5 mV. From Figure 4 it is obvious that $\Delta\psi$ is stable for at least 8 min, a time sufficient for performing a rotational diffusion measurement which requires ~5 min.

The imposition of $\Delta\psi$ can be prevented by addition of valinomycin at a molar ratio valinomycin/lipid = 1/500. As a further control, unlabeled lactose permease was reconstituted into vesicles of *E. coli* lipids and the suspension incubated for 15 min with a buffer containing 0.2 M KSCN. After sonication, substrate binding was measured and found to be unaffected by KSCN.

Sample preparation for rotational diffusion measurements

After reconstitution, the vesicle suspension was subjected three times to a freeze-thaw cycle between −20°C and 28°C in a plastic vessel. The size of the vesicles was examined by negative staining electron microscopy. Immediately after reconstitution, single vesicles of diameter 50 – 100 nm are found. The freeze-thaw cycles cause the vesicles to fuse and aggregate; one observes aggregates of dimension 500 nm consisting of small- and medium-sized vesicles, and a small number of larger vesicles up to 2 μm in diameter. The freeze-thawed samples were diluted to a lipid concentration of ~7 mM. For a permease/lipid molar ratio of 1/2500 this corresponds to a permease concentration of ~2.8 μM.

Prior to measuring, the solution was purged of oxygen by passing a stream of argon over the stirred sample for at least 15 min. This treatment was continued during the measurement. A membrane potential was imposed by adding 80 μl of KSCN in the appropriate buffer to 800 μl of the sample in 5 or 50 mM phosphate buffer, pH 7.5, followed by incubation for 3 min. The temperature dependency was measured by decreasing the temperature from high to low, with incubation for at least 15 min at each temperature. This temperature sequence was subsequently reversed in order to check for reversibility.

Three out of nine samples showed a rising phosphorescence anisotropy at longer times. The amplitude of the rising anisotropy varied from −0.05 to −0.26 with a relaxation time in the millisecond range. Such behavior was also observed on samples which had been incubated for several minutes at 95°C or boiled. It is known that boiling causes the permease to flocculate irreversibly and to lose the ability to bind substrate (Teather *et al.*, 1978). Thus, denaturation is likely to be the reason for a rising anisotropy. The samples exhibiting rising anisotropy were discarded. This elimination of samples supports the assumption made above that in the samples investigated the lactose permease possesses the native conformation.

Rotational diffusion measurements

Time-resolved phosphorescence measurements were performed on an instrument described previously (Austin *et al.*, 1979; Jovin *et al.*, 1981; Corin *et al.*, 1983). The excitation source, an excimer-pumped dye laser which delivers pulses of polarized light each of energy 3 mJ and duration 10 ns, was tuned to 515 nm. Emission was observed at 90° to the incoming light through a combination of cut-off filters centered at 520, 550 and 645 nm. This permits the observation of the eosin phosphorescence emitted at 690 nm. The intensities I_{δ} (t) and $I_{\parallel}$ (t) of the parallel and perpendicular polarized phosphorescence light were monitored through a sheet polarizer, which could be rotated by 90°, and detected by a photomultiplier followed by a transient recorder. Typically, 2048 light pulses were sampled for one measurement.

The high light intensity may cause bleaching of the dye and a corresponding loss of signal. Several measures were taken in order to minimize such losses: (i) the laser beam was expanded 5-fold in order to diffuse the light over as large a volume as possible; (ii) the samples were stirred continuously; and (iii) when necessary the laser beam was attenuated with neutral density filters until no decrease in signal could be observed during sampling of data.

The photomultiplier is electronically gated off just before the laser pulse for a total of 4 ns, but the gating is not perfect. As a consequence, the intense prompt fluorescence induces a spike in the photomultiplier signal which may last for up to 5 μs with the eosin concentrations used in this study. Hence, data analysis usually began 5 μs after the laser pulse in order to eliminate artifacts due to photomultiplier recovery.

In the data analysis, the phosphorescence intensities $I_{\parallel,\perp}$ (t) of a sample were first corrected for background phosphorescence by subtraction of the corresponding intensities of a reference sample. The reference samples did not contain eosin attached specifically to residue Cys 148 of the lactose permease. Their phosphorescence intensity was typically ~20% of that of the samples. From the corrected intensities $I^c_{\parallel,\perp}$ (t) the total phosphorescence intensity is constructed as $S(t) = I^c_{\parallel}(t) + 2I^c_{\perp}(t)$ and the phosphorescence anisotropy as $R(t) = [I^c_{\parallel}(t) - I^c_{\perp}(t)]/S(t)$. The curves S(t) and R(t) are fitted by a sum of exponentials using a Marquardt algorithm. In the case of the anisotropy, a constant term r_∞ is included so that the expression used for the fit is, assuming double-exponential relaxation,

$$r(t) = b_1 e^{-t/\phi_1} + b_2 e^{-t/\phi_2} + r_\infty. \quad (2)$$

The goodness of fit is examined by inspection of the residuals R(t) − r(t).

For the measurements within a time range of 200 μs three phosphorescence lifetimes of eosin were obtained, τ_1 = 1.9 μs, τ_2 = 18.7 μs and τ_3 = 141.2 μs. If measurements over longer times are fitted by three exponentials, life times of up to 2 ms are found.

Theory of rotational diffusion

In the theoretical treatment of rotational diffusion of membrane proteins, the proteins are described as cylinders with the cylinder axes parallel to the membrane normal. Rotation about this axis is governed by the diffusion coefficient $D_\parallel$, while rotation about an axis perpendicular to the cylinder axis is forbidden, i.e., $D_\perp = 0$ (Cherry, 1979). Then the phosphorescence anisotropy is given by (Tao, 1969):

$$r(t) = r_o[\tfrac{3}{4} \sin^4\theta\, e^{-4D_\parallel t} + 3 \sin^2\theta \cos^2\theta\, e^{-D_\parallel t} + \left(\frac{3\cos^2\theta - 1}{2} \right)^2] \quad (3)$$

with θ denoting the angle between the phosphorescence dipole moments, assumed to be the same for absorption and emission, and the cylinder axis. The theoretical value of the initial anisotropy if r_o = 0.4, if the chromophore orientation is fixed within the protein. If the chromophores, however, undergo some fast motion, r_o is smaller and the experimental value $r_o = b_1 + b_2 + r_\infty$ must be used. Equation 3 imposes severe restrictions on the amplitudes and relaxation times of equation 2, because b_1, b_2 ad r_∞ are determined solely by the angle θ,

$$b_1 = r_o \tfrac{3}{4} \sin^4\theta \quad (4a)$$

$$b_2 = r_o\, 3 \sin^2\theta \cos^2\theta \quad (4b)$$

$$r_\infty = r_o \left(\frac{3\cos^2\theta - 1}{2}\right)^2 \quad (4c)$$

and ϕ_1 and ϕ_2 are determined by the diffusion coefficient $D_\parallel$,

$$\phi_1 = (4D_\parallel)^{-1} \quad (5a)$$

$$\phi_2 = D_\parallel^{-1} \quad (5b)$$

The diffusion coefficient $D_\parallel$ depends on the viscosity $\eta_\parallel$ of the lipid phase for rotation about the membrane normal and on the size of the protein specified by

the effective volume V_c according to (Saffman and Delbrück, 1975)

$$D_{\parallel} = \frac{kT}{4\eta_{\parallel} V_c} \nu \quad (6)$$

with $\nu = 1$ and

$$V_c = \pi a^2 h, \quad (7)$$

a denoting the cylinder radius and h the height of the cylinder equal to the thickness of the membrane. Hence, if $D_{\parallel}$ is measured, either the viscosity $\eta_{\parallel}$ or the effective protein radius a can be derived.

For the case of bacteriorhodopsin in DMPC at 28°C, Cherry and Godfrey (1981) obtained $\phi_2 = D_{\parallel}^{-1} = 15 \pm 5$ μs. The spatial dimensions of bacteriorhodopsin are ~25 Å × 35 Å × 45 Å (Henderson and Unwin, 1975), the latter value for the height h being roughly equal to the thickness of the DMPC membrane. In evaluating $D_{\parallel}$ for the viscosity $\eta_{\parallel}$, Cherry and Godfrey made the assumption that the effective protein radius is determined by the larger cross-sectional dimension of 35 Å, so that $a = 17.5$ Å. Using equation 6, they obtained $\eta_{\parallel} = 3.7 \pm 1.3$ poise.

The assumption about the effective protein radius may be questioned, however, because a cylinder is expected to rotate faster than an object with a flattened cross-section. The diffusion coefficient $D_{\parallel}$ of a rod with an elliptic cross-section of half axes a_1 and a_2 can be derived from the work of Perrin (1934) on the rotational diffusion of an arbitrary ellipsoid and results as in equation 6 with the shape factor ν, given in terms of the axial ratio $\gamma = a_1/a_2 \geq 1$,

$$\nu = \frac{2\gamma}{1 + \gamma^2} \quad (8)$$

and $V_c = \pi a_1 a_2 h$.

Before using equation 6 to determine $\eta_{\parallel}$, the spatial dimensions of bacteriorhodopsin which are given above and lead to $V_c = \pi\ 17.5 \times 12.5 \times 45$ Å^3 = 30 925 Å^3 may be slightly refined to be consistent with the actual volume V = 33 100 Å^3, derived from the mol. wt. 26 800 and the partial specific volume 0.74 cm^3/g. This is accomplished by using the values $a_1 = 18$ Å and $a_2 = 13$ Å leading to $\nu = 0.95$. With these values, the diffusion coefficient $D_{\parallel}$ measured by Cherry and Godfrey and inserted into equation 6 yields $\eta_{\parallel} = 4.5 \pm 1.5$ poise for DMPC at 28°C.

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