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LOCALIZATION OF THE GALACTOSIDE BINDING SITE IN THE LACTOSE CARRIER OF *ESCHERICHIA COLI*

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The location of fluorophores specifically bound to the lactose/H^+ carrier of *Escherichia coli* was ascertained by the use of various collisional quenchers. The reporter groups were (1) the pyrenyl residue of *N*-(1-pyrenyl)maleimide attached to the essential cysteine residue 148, which is presumably at or near the galactoside binding site, and (2) the dansyl moieties of a series of fluorescent substrate molecules. The accessibility of these fluorophores from the lipid phase was assessed by nitroxyl-labelled fatty acids and phospholipids. By using a series of nitroxyl-labelled fatty acids carrying the quencher at different positions in the acyl chain, the position of a quenchable fluorophore with respect to the membrane normal can be determined. The accessibility of fluorophores from the aqueous phase was assessed by using a water-soluble quencher, the *N*-methylpicolinium ion. The results of quenching studies suggest that the galactoside binding site is located within the carrier and that this binding site communicates with the aqueous phase through a pore.

Introduction

The lactose/H^+ carrier of *Escherichia coli* is a protein of the cytoplasmic membrane responsible for the active transport of galactoside. The carrier acts as a symporter; the transport of galactosides is strictly coupled to the concomitant transport of H^+ (for recent reviews see Refs. 1 and 2). The amino acid sequence of the carrier is known and consists of 417 residues [3,4]. Models for the folding of the carrier based upon the sequence indicate that 12–14 bilayer-spanning α-helices may be present (Ref. 5; Beyreuther in Ref. 6). Circular dichroism measurements on the purified, reconstituted carrier reveal a high α-helix content [5].

A significant refinement of the structural models could be achieved if the galactoside binding site could be located. Two types of reporter groups are suited for such a study. Firstly, among the large number of galactosides which are bound and transported is a series of fluorescent dansyl galactosides [7]. These can be used to locate the binding site employing spectroscopic techniques.

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Abbreviations: lactose, 4-*O*-β-D-galactosyl-α-D-glucose; GalSGal, β-D-galactosyl 1-thio-β-D-galactoside; DnsEtOGal, 2′-*N*-dansylaminoethyl 1-*O*-β-D-galactoside; DnsHxSGal, 6′-*N*-dansylaminohexyl 1-thio-β-D-galactoside; MalNEt, *N*-ethylmaleimide; MalNPyr, *N*-(1-pyrenyl)maleimide; MalNPyr-carrier, MalNPyr-labelled lactose carrier; $ClHgBzSO_3$, *p*-chloromercuribenzene sulfonate, sodium salt; MePic, *N*-methylpicolinium ion; $1NOC_{12}$, 4-dodecanoyloxy-2,2,6,6-tetramethylpiperidine-1-oxyl; $5NOC_{18}$, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl; $12NOC_{18}$, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl; 5NOPC, L-α-phosphatidylcholine stearoyl $5NOC_{18}$.

Secondly, galactoside binding and transport are inhibited by sulfhydryl reagents and, inversely, the carrier can be protected against most such reagents by substrate binding [8,9]. Recently, Cys-148 has been identified as the site of modification [10]. Hence, the simplest assumption is that Cys-148 must lie in the vicinity of the substrate binding site. Thus, fluorescent groups attached to Cys-148 can be used to approximately locate the binding site.

The advantage of using fluorescent galactosides is that, by definition, these substrates probe the binding site. The disadvantage is that there is a high background fluorescence and that galactoside binding is sensitive to a wide variety of compounds [9,11–13]. The advantage of using carrier covalently labelled with a fluorophore is that the background is low. However, the localization of Cys-148 in or near the binding site is indirectly inferred from the substrate protection.

Our strategy is to assess the accessibility of these two types of reporter groups from the aqueous and lipid (membrane) phases. A diffusional (collisional) encounter between the fluorescent reporter groups and certain molecules leads to a quenching of the fluorescence which is designated dynamic. Dynamic quenching results in a decrease in the intensity of the steady-state fluorescence and in the fluorescent lifetime of the fluorophore, as judged by time-resolved fluorescence measurements. The accessibility of fluorophores from the aqueous phase is investigated by employing as a dynamic quencher the *N*-methylpicolinium ion (MePic) [14]. The accessibility from the lipid phase is investigated by employing a series of lipophilic nitroxides, which are commonly used as spin labels in electron spin resonance but have also been demonstrated to function as dynamic quenchers [15]. Because these compounds position the quencher at different depths in the bilayer, the position of the fluorophore along the membrane normal may be determined [16,17].

Materials and Methods

Chemicals. The substrates dansylaminoethyl *O*-β-D-galactoside (DnsEtOGal) and dansylaminohexyl thio-β-D-galactoside (DnsHxSGal) were synthesized according to the method of Schuldiner et al. [18]. Radioactive labelling of galactosides was accomplished as described by Kennedy et al. [11]. *N*-Methylpicolinium perchlorate was a gift from P. Overath (Max-Planck-Institut für Biologie, Tübingen, F.R.G.). *N*-Ethylmaleimide (MalNEt), diatomaceous earth, *p*-chloromercuribenzene sulfonate ($ClHgBzSO_3$), and stearic acid were obtained from Sigma Chemical Co. (Munich, F.R.G.). *N*-(1-pyrenyl)Maleimide and dipalmitoylphosphatidylcholine (DPPC) were purchased from Fluka (Heidelberg, F.R.G.). 3-(1-pyrenyl)Butanoate was a product of Eastman Kodak (Heidelberg, F.R.G.). 4-Dodecanoyloxy-2,2,6,6-tetramethylpiperidine-1-oxyl ($1NOC_{12}$), 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl ($5NOC_{18}$), and 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl ($12NOC_{18}$) were obtained from Syva Research Chemicals (Palo Alto, CA). L-α-Phosphatidylcholine stearoyl $5NOC_{18}$ (5NOPC) was a gift from D. Marsh (Max-Planck-Institut für biophysikalische Chemie, Göttingen, F.R.G.).

Preparation of membrane vesicles and measurement of substrate binding. Cytoplasmic membrane vesicles were derived from the carrier overproducing strain T206 as previously described [12,19]. The binding of *p*-nitrophenyl α-D-galactoside and DnsEtOGal was measured by flow dialysis [12] and was in the range 3–4 nmol/mg membrane protein. For fluorescence measurements vesicles were suspended in 100 mM potassium-hydrogen phosphate, pH 6.5, by sonication. The protein concentration was 0.7–1.0 mg/ml for studies of galactosides and 0.05 mg/ml for pyrene fluorescence measurements. Buffer was saturated with N_2 to reduce the fluorescence quenching due to O_2. For fluorescence quenching with lipophilic quenchers, appropriate amounts of a 10 mM solution of nitroxides in ethanol were added to the vesicle preparation and incubated for 30 min at room temperature. The nitroxides attached either to fatty acids or lipids incorporate spontaneously into the membranes [20].

Partition coefficient of MePic. The partitioning of MePic between isobutanol and water was measured to simulate the partitioning between the membrane and water [21]. A mixture of isobutanol and water containing 0.1 mM MePic perchlorate was shaken for 10 min and the phases were allowed to separate overnight. The concentration of

MePic in both solvents was determined from the absorption spectra [14].

Preparation of lactose carrier labelled with MalNPyr (MalNPyr-carrier). Cytoplasmic membrane vesicles prepared from the carrier-overproducer T206 were incubated with MalNEt and iodoacetamide in the presence of GalSGal to block reactive thiol groups other than the essential cysteinyl residue in the lactose carrier. These vesicles are referred to as pretreated membranes [12].

10 mg (34 μmol) MalNPyr were dissolved in 5–10 ml benzene, 1 g diatomaceous earth (97.5% SiO_2) was added, and the suspension was sonicated for 5 min. Benzene was removed under a stream of N_2. 5 ml diethylether were added, the suspension was stirred with a glass rod, and the solvent was removed under a stream of N_2. Traces of solvent were removed by allowing the diatomaceous earth to remain at least 1 h in the dark at 37 °C.

To 2 ml pretreated membrane (5 mg/ml) in 50 mM potassium-hydrogen phosphate (pH 6.3) were added 20–100 mg MalNPyr-diatomaceous earth (about 0.3–1.6 mM MalNPyr). The suspension was slowly stirred for 45 min at 20 °C and was then diluted to 5 ml with 50 mM potassium-hydrogen phosphate/1 mM cysteine (pH 6.3) and sonicated 5 min. The suspension was layered upon 1.5 ml 20% sucrose and centrifuged for 15 min at $3000 \times g$ to remove the diatomaceous earth. The diatomaceous earth was resuspended in 5 ml buffer, sonicated, and recentrifuged as above. The supernatants from the two centrifugation steps containing the labelled vesicles were diluted to 40 ml with buffer, and centrifuged for 90 min at $120000 \times g$. The vesicles were washed twice in 40 ml 100 mM sodium 5-sulfosalicylate/10 mM Tris/1 mM EDTA (pH 7.5) after sonication. Subsequently, vesicles were washed twice in water and once in the appropriate buffer. Vesicles for control experiments were prepared by subjecting membranes from uninduced T206 (i.e., containing no lactose carrier) to the above treatment or by subjecting pretreated membrane to treatment with MalNEt (thus blocking the essential cysteine residue) before labelling with MalNPyr.

Steady-state fluorescence measurement. Steady-state fluorescence was detected by a Perkin-Elmer MPF-3 spectrofluorimeter at an angle of 90° using a 1×1 cm cuvette, thermostatted at 20 °C. Fluorescence emission spectra were recorded at the excitation maxima at 345 nm for MalNPyr-carrier and at 360 nm for the specifically bound DnsEtOGal and DnsHxSGal. Most quenching experiments were performed at fixed emission wavelengths of 375 nm for MalNPyr and 490 nm for DnsEtOGal. Steady-state measurements of the fluorescence quenching were carried out by titration. Small amounts of concentrated quencher solution (10 mM for lipid-soluble quenchers and 1 M for MePic) were added successively to the membrane suspension, and the fluorescence intensity was measured after equilibrium had been reached. Samples were magnetically stirred continuously.

Time-resolved fluorescence measurement. The time-resolved fluorescence measurements were carried out with a pulsed laser system (Spectra Physics) and conventional single-photon-counting equipment. The pulse repetition rate was 0.8 MHz, and the counting rate of single photons was set to about 8 kHz. The excitation wavelength was 317 nm and the emission wavelength selected by a monochromator was 375 nm.

The exciting light was polarized perpendicular to the plane defined by the excitation and emission. A sheet polarizer set at the magic-angle to the polarization of the exciting light was inserted into the emission beam.

The decay curve was analyzed by deconvolution, assuming three lifetimes

$$i(t) = \sum_{i=1}^{3} a_i \exp\left(-\frac{t}{\tau_i}\right) \tag{1}$$

with

$$\sum_{i=1}^{3} a_i = 1.$$

Then, the dynamic part of the quenching was estimated comparing the average lifetime

$$\langle \tau \rangle = \int i(t)\mathrm{d}t = \sum_{i=1}^{3} a_i \tau_i$$

in the presence and absence of quencher.

Results

Specific labelling of the lactose carrier with MalNPyr

When cytoplasmic membranes from the carrier-overproducer strain T206 are treated with MalNEt and iodoacetamide in the presence of saturating levels of the substrate GalSGal, the essential cysteine residue is protected, whereas all other accessible cysteine residues react with MalNEt or iodoacetamide. To be certain that all thiol groups other than the essential cysteine residue are blocked by the pretreatment with MalNEt and iodoacetamide in the presence of GalSGal, the incubation time was increased. This results in the loss of 29% of the original binding sites, but the subsequent labelling of cysteine residues is very specific, as indicated by two observations. The subsequent incorporation of radioactive MalNEt can be reduced by 91% in the presence of GalSGal, demonstrating that the essential residue in the lactose carrier is labelled. Carrier-lacking membranes, when pretreated according to the described method, incorporate only 11% as much radioactive MalNEt as carrier-containing membranes. These results imply that about 90% of the incorporated maleimide is located in the carrier [12].

When pretreated membranes are treated with MalNPyr dispersed on diatomaceous earth and washed in 5-sulfosalicylate in the presence of cysteine, the galactoside binding activity decreases, indicating that the essential cysteine residue is modified, and, after electrophoretic separation in the presence of sodium dodecyl sulfate, the lactose carrier exhibits pyrene fluorescence. If labelling is performed by MalNPyr dissolved in organic solvents, a significant pyrene fluorescence is observable at the front (data not shown).

The fluorescence emission spectrum of MalNPyr-labelled, pretreated membranes exhibits three maxima at 377, 395, and 416 nm (Fig. 1), in accordance with previously published spectra of MalNPyr-labelled proteins [22,23]. No excimer fluorescence around 480 nm is visible, suggesting that no direct pyrene-pyrene or assumedly binding site-binding site contacts are present. The purified lactose carrier was demonstrated to be a monomer in detergent micelles, and no evidence of negative dominance could be observed in a survey of

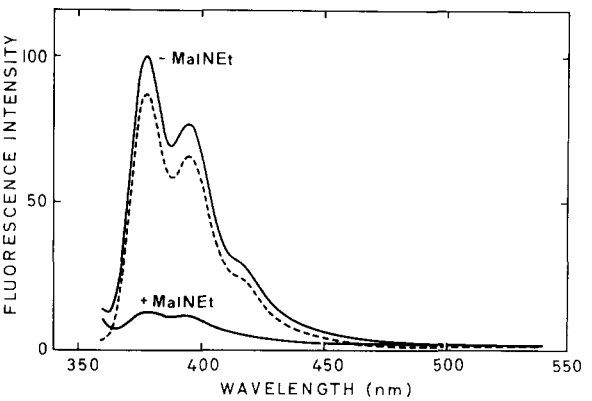

Fig. 1. Fluorescence emission spectra of MalPyr-labelled, pretreated T206 vesicles. Pretreated vesicles are labelled directly with MalNPyr (−MalNEt) or after treatment with MalNEt (+MalNEt) to block the essential cysteine residue in the carrier. The difference in these spectra yields the contribution of the specifically labelled MalNPyr-labelled carrier (dashed line). The excitation wavelength is 345 nm.

mutants [24]. These observations suggest that the lactose carrier is probably a monomer under these experimental conditions.

If pretreated membranes are exposed to MalNEt in the absence of GalSGal and subsequently treated with MalNPyr, the pyrene fluorescence of the membranes is reduced to 15% of that of pretreated membranes labelled with MalNPyr. After correcting for the light scattering or the suspension, about 10% of the pyrene fluorescence is attributed to nonspecific binding to the membranes (Fig. 1). Similar results are obtained when vesicles derived from cells uninduced for the synthesis of the lactose carrier are pretreated and then exposed to MalNPyr (data not shown).

Accessibility of pyrenyl moiety from the lipid phase

Addition of $5NOC_{18}$ to MalNPyr-labelled vesicles results in a quenching of the pyrene fluorescence (Fig. 2a). This quenching must be dynamic (i.e., the result of a diffusional encounter between the pyrenyl moiety and $5NOC_{18}$) and not static in nature (i.e., the result of the formation of a stable complex between the pyrenyl moiety and $5NOC_{18}$), as the same dependence of the quenching upon the $5NOC_{18}$ concentration is observed in the presence of 40 or 180 μM stearic acid (Fig. 2a). Thus, stearic acid and $5NOC_{18}$ do not compete for

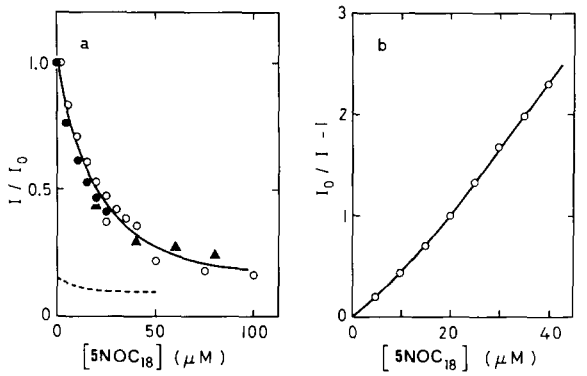

Fig. 2. Fluorescence quenching of MalNPyr-carrier by $5NOC_{18}$. (a) The fluorescence intensity is plotted as a function of the $5NOC_{18}$ concentration in the presence of 0 (○), 40 (●), and (▲) 150 μM stearic acid. The dashed line represents the fluorescence from the control vesicle preparation (see Fig. 1: + MalNEt). (b) The fluorescence quenching of the specific MalNPyr-carrier fluorescence presented in a Stern-Volmer plot.

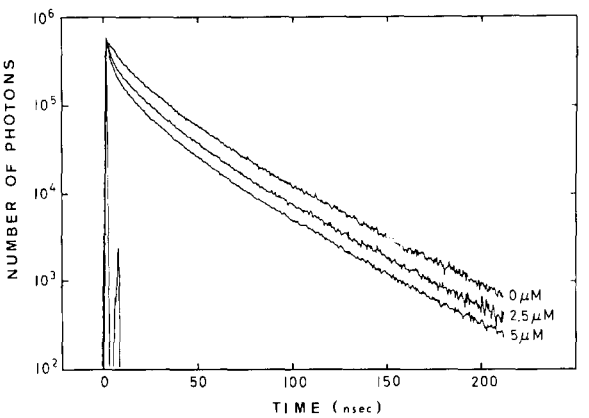

Fig. 3. Time-resolved fluorescence measurements of MalNPyr-carrier. The curves for the decay of the specific MalNPyr-carrier fluorescence are obtained in the presence of 0, 2.5, and 5.0 μM $5NOC_{18}$. The exciting light pulse appears on the left.

a binding site next to the pyrenyl moiety, and the quenching of the fluorescence of MalNPyr-carrier by $5NOC_{18}$ is due to collisions between $5NOC_{18}$ and the fluorophore. The effects of $5NOC_{18}$ on the nonspecific background fluorescence is also indicated (Fig. 2a).

The quenching of the specific MalNPyr-carrier fluorescence by $5NOC_{18}$ evinces a nearly linear concentration dependence when analyzed in a Stern-Volmer plot (Fig. 2b),

$$\frac{I_0}{I} - 1 = Kc \qquad (2)$$

where I_0 is the initial fluorescence intensity, I is the intensity at the concentration c of quencher, and K is the Stern-Volmer constant. The absence of saturation shows that all fluorophores are equally quenchable or that all pyrenyl groups, at or near the galactoside binding site, are accessible to 5 NOC_{18}.

Time-resolved fluorescence measurements are performed to confirm the collisional origin of the fluorescence quenching. In the case of dynamic quenching, the fluorescence decays more rapidly in the presence of quencher, whereas in the case of static quenching the time course of the decay is unaffected by the presence of quencher [25]. In the absence of quencher, the time-resolved fluorescence decay of MalNPyr-carrier can be described by two lifetimes, 31 and 105 ns (Fig. 3). These values agree with results from the literature [22] and with our own measurements of the lifetimes of pyrene in lipid membranes (unpublished data), indicating that the existence of two lifetimes does not imply the presence of two species of carrier-bound pyrenyl groups. For the purpose of subsequent comparisons, an average lifetime $\langle\tau\rangle$ is computed by fitting decay curves with three lifetimes in the presence and absence of quencher. The fluorescence decay is more rapid in the presence of $5NOC_{18}$ (Fig. 3). Without quencher the three lifetimes, whose amplitudes are of comparable magnitude are 14, 51 and 118 ns, leading to $\langle\tau\rangle = 61$ ns. In the presence of 5 μM $5NOC_{18}$ they decrease to 7, 41 and 107 ns, with $\langle\tau\rangle = 39$ ns. For technical reasons, the shortest lifetime is the least reliable one. The other two agree closely with the original two lifetimes of pyrene, and they decrease uniformly upon addition of quencher, consistent with the notion that the carrier is labelled by one pyrene group.

Fluorescence quenching is generally the sum of the contributions from the static and dynamic mechanisms. The individual contributions may be estimated by comparing the time-resolved and steady-state measurements. The Stern-Volmer constant K from the steady-state measurement is the sum of the Stern-Volmer constants from the dy-

namic quenching K_d and the static quenching K_s:

$$K = K_d + K_s \tag{3}$$

On the other hand, the Stern-Volmer plot of the time-resolved measurements reflects the dynamic quenching alone,

$$\frac{\langle\tau\rangle_0}{\langle\tau\rangle} - 1 = K_d c \tag{4}$$

where $\langle\tau\rangle$ is the average lifetime. For pure static quenching $(\langle\tau\rangle_0/\langle\tau\rangle) - 1 = 0$, whereas for pure dynamic quenching $(\langle\tau\rangle_0/\langle\tau\rangle) - 1$ is equal to $(I_0/I) - 1$. Therefore, the analysis of the data in Fig. 4 indicates that the fluorescence quenching of MalNPyr-carrier by $5NOC_{18}$ is completely dynamic, in accord with the observation of no inhibition of the quenching by stearic acid.

In principle, there are two possible modes of dynamic quenching through collisions between $5NOC_{18}$ and the pyrenyl group at the binding center: 5NOC18 can diffuse through water or through the lipid phase. However, because fatty acids partition preferentially into the lipid phase,

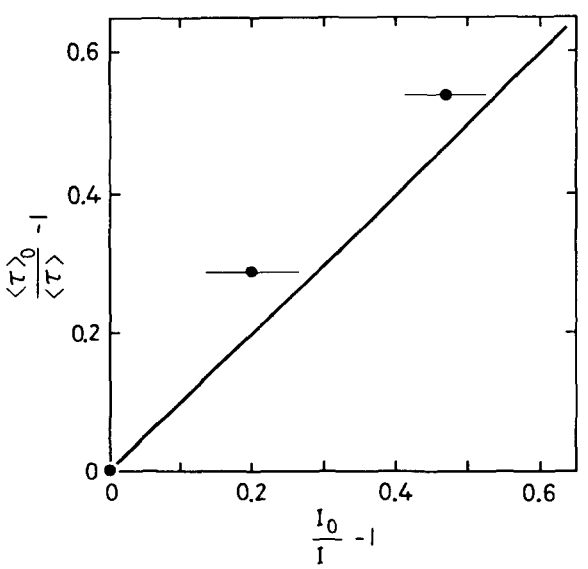

Fig. 4. Confirmation of the dynamic nature of the quenching of MalNPyr-carrier fluorescence by $5NOC_{18}$. The average fluorescence life times $\langle\tau\rangle$ were obtained from time-resolved measurements (Fig. 3) and the steady-state intensities refer to the same sample. The straight line with a slope of unity corresponds to completely dynamic quenching.

collisional encounters between the pyrenyl group and $5NOC_{18}$ in the lipid phase are expected to be the dominant process. This argument can be quantitatively formulated in the following way. For diffusion through water the Stern-Volmer constant is given by the Smoluchowski relation.

$$K_d = 4\pi R \tau_0 D \tag{5}$$

where R is the capture radius, τ_0 the lifetime in the absence of quencher, and $D = D_{\text{quencher}} + D_{\text{fluorophere}}$ the effective diffusion constant. Using $\tau_0 = 60$ ns and as a typical value $R = 10$ Å, the experimental Stern-Volmer constant of $K_d = 5 \cdot 10^4\ M^{-1}$ yields $D = 10^{-3}\ cm^2/s$. Compared to realistic values for diffusion in water, this number is two orders of magnitude too high. This discrepancy would become even larger if the high preference of the fatty acid quenchers for the lipid phase and the corresponding lower quencher concentration in the water phase were taken into account. For diffusion through the lipid phase, the dependence of I_0/I on the quencher concentration is not merely linear as predicted by the Stern-Volmer expression Eqn. 2, but given approximately (Owen [26]) by

$$\frac{I_0}{I} = \frac{1 + 1.58 Z f c \tau_0 D}{1 - 3.54 Z f c R \sqrt{\pi \tau_0 D}} \tag{6}$$

where Z is the thickness of the membrane and f the ratio of total volume to membrane volume. Using $Z = 40$ Å and $f = \frac{1}{3} \cdot 10^5$ for the fatty acid quenchers used, Eqn. 6 can be fitted to the quenching data of Fig. 2 yielding $R \approx 0.5$ Å and $D \approx 10^{-7}\ cm^2/s$. Such a value for the diffusion constant is typical for fatty acids in membranes, whereas the value for the capture radius is remarkably small. This may indicate that the pyrenyl group is not equally accessible to quencher molecules approaching from every direction in the plane of the membrane, but only to those approaching from a limited angular range, thus rendering the effective capture radius small. Such a limited accessibility would imply that the pyrenyl group is to a large extent shielded by parts of the lactose carrier from the lipid phase. The quantitative comparison between quencher diffusion through water and through lipid suggests that the fatty acid quenchers reach the MalNPyr-carrier from the lipid phase.

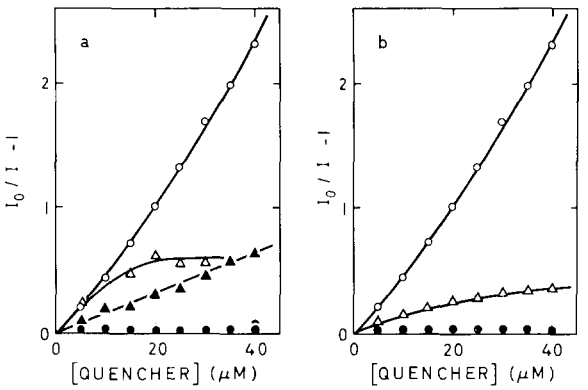

Fig. 5. Quenching of MalNPyr-carrier fluorescence by various hydrophobic nitroxides. (a) Stern-Volmer plot of quenching by $1NOC_{12}$ (△), $5NOC_{18}$ (○), $12NOC_{18}$ (▲), and stearic acid (●, control). (b) Stern-Volmer plot of quenching by $5NOC_{18}$ (○), 5NOPC (△), and dipalmitoyl L-α-phosphatidyl choline (●, DPPC; control).

This point is underscored by the observation that a nitroxide attached to a phospholipid also quenches the MalNPyr-carrier (Fig. 5b). The preference of a phospholipid for the lipid phase is even higher than for a fatty acid, so that diffusion through the water phase can be excluded in this case. The lower quenching efficiency of the phospholipid quencher 5NOPC as compared to that for $5NOC_{18}$ must be due to a reduced rate of collisions between 5NOPC and the pyrenyl group. Only one of the two acyl chains in 5NOPC carries the nitroxyl group, and the diffusion of the bulkier 5NOPC may be about a factor of 2 slower than the diffusion of $5NOC_{18}$. Thus the rate of collision leading to quenching may be lower by a factor of 4 for 5NOPC compared to $5NOC_{18}$, which is sufficient to explain the observed difference in quenching efficiency.

Position of the pyrenyl moiety on the membrane normal

Once the dynamic nature of the quenching is verified, the ability of a series of quenchers with the nitroxide function at different positions along the chain to quench the fluorophore in MalNPyr-carrier may be contrasted. $1NOC_{12}$, $5NOC_{18}$ and $12NOC_{18}$ quench the fluorescence of the pyrenyl moiety to different extents (Fig. 5a). When the nitroxyl group is at the height of carbon 5 in the lipid chain, quenching is most efficient. However, when the nitroxyl group is at the C-1 or C-12 position quenching is less pronounced. As mentioned above, stearic acid has no effect upon the fluorescence. To interpret such observations, the positions along the hydrocarbon chains are assumed equivalent to positions along the membrane normal [17]. In the case of $12NOC_{18}$ this assumption is not trivial, because the C_{18} chain could fold back so that the nitroxyl group lies at the lipid-water interface. This possibility, however, is ruled out by quenching experiments on a synthetic polypeptide which spans the lipid bilayer as an α-helix with a tryptophan residue in the middle of the helix. In this case, $12NOC_{18}$ was found to be the most efficient quencher (Hartman, P., Vogel, H. and Jähnig, F., unpublished data) *. These results lead to the conclusion that the pyrenyl moiety in MalNPyr-carrier is located at a height in the protein corresponding approximately to the C-5 position in the lipid chain and is accessible from the lipid phase.

Accessibility of the pyrenyl moiety from the aqueous phase

The ability of a water-soluble compound such as *N*-methylpicolinium (MePic) to quench the fluorophore in MalNPyr-carrier is a measure of the accessibility of the fluorophore from the aqueous phase. MePic effectively quenches the fluorescence of 3-(1-pyrenyl)butanote in aqueous solution (Fig. 6). In contradistinction, the fluorophore in MalNPyr-carrier is much less efficiently quenched by MePic, as judged from the 50-fold smaller Stern-Volmer constant (Fig. 6). The simplest way to explain this difference would be in terms of an immobilization of the fluorophore if membrane-bound to the carrier. In the extreme case of complete immobilization, however, this would yield only a reduction by a factor of 2 in the effective diffusion constant and consequently in the Stern-Volmer constant K_d, Eqn. 5. Another possible

* The curvature in the Stern-Volmer plot for $1NOC_{12}$ is not understood at present. This unusual behavior may be attributable to the absence of a charged group in this compound. Thus $1NOC_{12}$ may not be as firmly anchored at the water-membrane interface as the other quenchers.

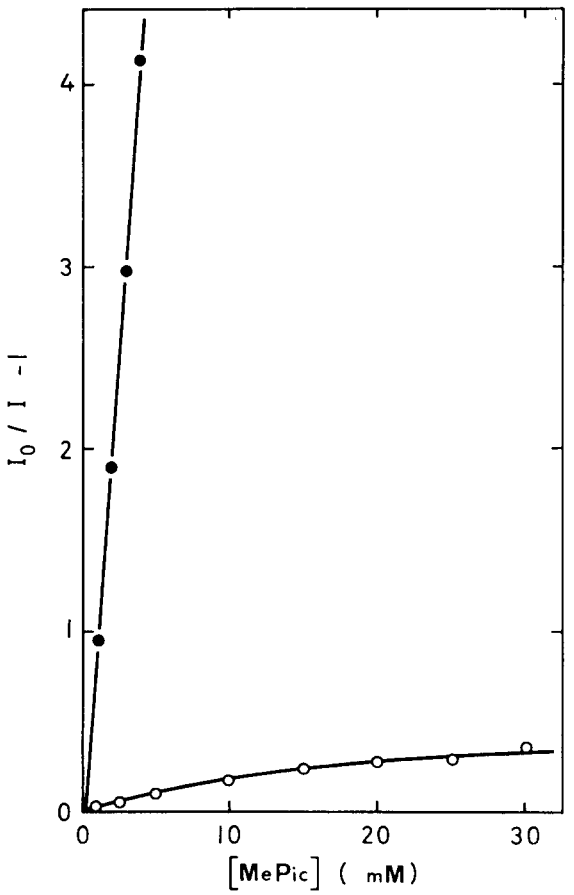

Fig. 6. Stern-Volmer plots of the quenching of MalNPyr-carrier (○) and 3-(1-pyrenyl)butanoate in water (●) by the water-soluble quencher MePic.

interpretation would be that positive charges of the exposed carrier surface could repel the positively charged MePic. However, in that case MePic should also be ineffective in quenching specifically bound dansylgalactosides, contrary to the experimental finding (see below). Therefore, the weaker quenching of the pyrenyl group in MalNPyr-carrier as compared to that of the fluorophore in aqueous solution suggests that the fluorophore is effectively sequestered from the aqueous phase in the former case.

An attempt was made to determine the origin of the small but measurable quenching of the fluorophore in MalNPyr-carrier. The partition coefficient of MePic between isobutanol and water is 0.13 ± 0.03, which represents an upper limit for

TABLE I

EFFECTIVE DIFFUSION CONSTANTS FOR FLUORESCENCE PROBES AND QUENCHERS

(a) D calculated for diffusion in the membrane, Eqn. 6. (b) D calculated for diffusion in water, Eqn. 5, assuming $R = 10$ Å.

Fluorophore	Quencher	K_d (M^{-1})	τ_0 (ns)	D (cm^2/s)
MalNPyr-carrier	$5NOC_{18}$		61	$1 \cdot 10^{-7}$ (a)
MalNPyr-carrier	$12NOC_{18}$		61	$3 \cdot 10^{-8}$ (a)
MalNPyr-carrier	MePic	20	61	$4 \cdot 10^{-7}$ (b)
Pyrenylbutanoate	MePic	1000	98	$1 \cdot 10^{-5}$ (b)
DnsEtOGal (in water)	MePic	18	3	$8 \cdot 10^{-6}$ (b)
DnsEtOGal (carrier-bound)	MePic	28	20	$2 \cdot 10^{-6}$ (b)
DnsHxSGal (carrier-bound)	MePic	18	20	$1 \cdot 10^{-6}$ (b)

the partition coefficient of this compound between the membrane and water [21]. Employing Eqn. 5 or 6 with $f = 0.13$ to derive the apparent diffusion constants characteristics for quenching from the aqueous phase or from the lipid phase, the apparent diffusion constant for quenching from the lipid phase is unrealistically high. This indicates that quenching occurs preferentially from the aqueous phase (Table I). Thus, MePic quenching of the fluorophore is apparently due to the diffusion of MePic from the aqueous phase to the pyrenyl residue. This diffusion, however, is hindered by the protein matrix of the carrier, in comparison to the free diffusion of MePic in water.

Spectroscopic properties of carrier-bound dansyl galactosides

If a dansyl galactoside is added to a suspension of T206 membranes, its partitioning into the membrane and binding to the carrier results in three different species: dansyl galactoside specifically bound to the carrier, unspecifically bound to the membrane, and dissolved in water [7]. Their individual contributions to the fluorescence signal can be determined, as exemplified in Fig. 7, which shows four kinds of spectra for the case of DnsEtOGal. The spectrum of specifically bound DnsEtOGal is obtained from the difference between the spectra of membranes with DnsEtOGal in the

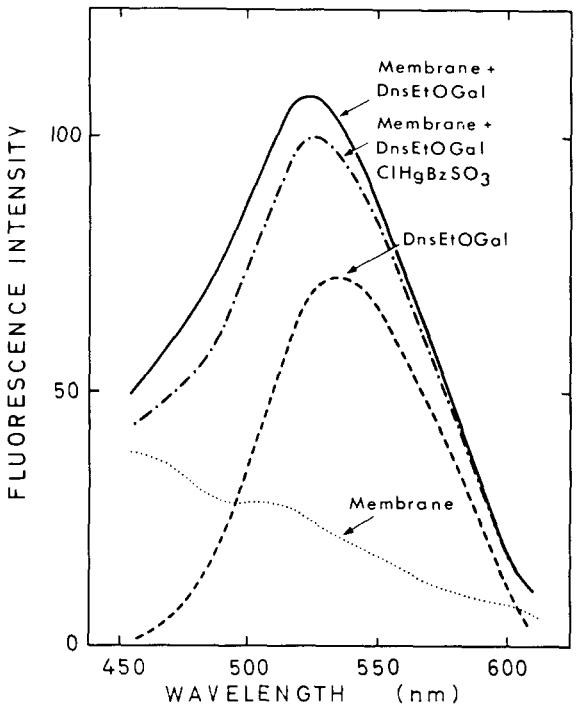

Fig. 7. Fluorescence emission spectra of the substrate DnsEtOGal (20 μM) in water (— — —), in the presence of 0.8 mg/ml T206 membranes (————), and in the presence of 0.8 mg/ml T206 membranes where substrate binding is inhibited by 0.4 mM $ClHgBzSO_3$ (·-·-·). The fluorescence is excited at 360 nm in all cases.

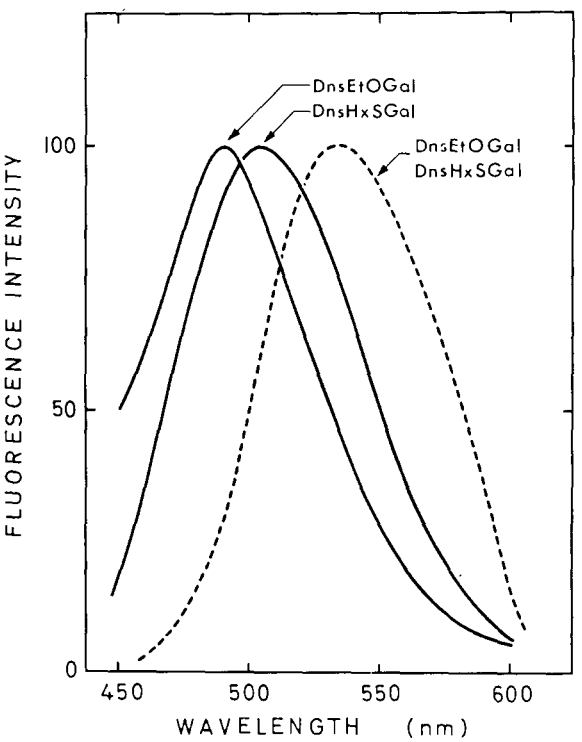

Fig. 8. Fluorescence emission spectra of DnsEtOGal and DnsHxSGal bound to the lactose carrier (————) and dissolved in water (— — —, superimposed).

absence and presence of $ClHgBzSO_3$, which inhibits the specific binding of DnsEtOGal. The spectrum of unspecifically bound DnsEtOGal is obtained if from the spectrum of membrane with DnsEtOGal the spectra of membrane alone, specifically bound DnsEtOGal, and DnsEtOGal in water are subtracted. The same analysis can be performed for DnsHxSGal.

The fluorescence emission spectra of the specifically bound DnsEtOGal and DnsHxSGal are compared in Fig. 8, along with the spectrum of both the galactosides in water. The emission maxima are shifted to shorter wavelengths, indicating that the environment of the dansyl groups becomes more hydrophobic upon binding to the carrier. The same conclusion can be drawn from the concomitant increase of the fluorescence intensity (data not shown) and of the fluorescence lifetime (Table I). By comparison with the fluorescence characteristics of dansyl galactoside in various dioxane/water mixtures of known dielectric constant, the dansyl group of specifically bound DnsEtOGal was found to sense an environment of dielectric constant 2 [7]. In the case of specifically bound DnsHxSGal the environment is slightly more polar, corresponding to a dielectric constant of 4.

Accessibility of dansyl galactosides

To study the accessibility of the carrier-bound fluorophore, the effect of quenchers in the aqueous or lipid phase upon the fluorescence was investigated. As, however, the dansyl galactosides are not covalently attached to the carrier as is the case with MalNPyr, direct effects of the quenchers upon substrate binding must be assessed parallel to the fluorescence measurements. Addition of the fatty acid quencher $5NOC_{18}$ to membranes in the presence of DnsEtOGal results in a decrease in the fluorescence of the specifically bound DnsEtOGal

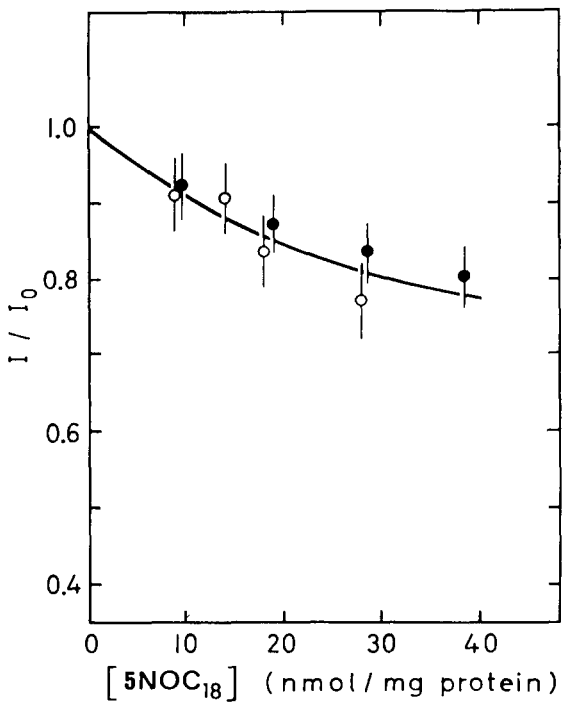

Fig. 9. Fluorescence intensity of carrier-bound DnsEtOGal as a function of $5NOC_{18}$ concentration (●). The change in the fluorescence intensity calculated from the observed inhibition of DnsEtOGal binding by $5NOC_{18}$ is indicated (○).

(Fig. 9). When the binding of labelled DnsEtOGal to the carrier is independently measured, addition of $5NOC_{18}$ reduced the amount of galactoside bound to the carrier. Plotting these data in a manner analogous to that used for analyzing the decrease in fluorescence (Fig. 9), the effect of $5NOC_{18}$ upon the fluorescence can be completely attributed to perturbation of the galactoside binding. After correcting for this effect, the fluorescence of carrier-bound DnsEtSGal is not quenched by $5NOC_{18}$, leading to the conclusion that the dansyl group is not accessible to this quencher from the lipid phase. This same conclusion was obtained for both DnsEtOGal and DnsHxSGal with all lipid-soluble quenchers, $1NOC_{12}$, $5NOC_{18}$, $12NOC_{18}$ and 5NOPC (data not shown).

The accessibility of carrier-bound DnsEtOGal from the aqueous phase is assessed by the effects of MePic. The fluorescences of aqueous DnsEtOGal and of carrier-bound DnsEtOGal are quenched by MePic with Stern-Volmer constants of 18 M^{-1} and 28 M^{-1}, respectively (Table I). MePic does not inhibit the binding of labelled DnsEtOGal to the carrier, indicating that the decrease in fluorescence of the carrier-bound galactoside is not due to inhibition of substrate binding. The fluorescence lifetimes of aqueous and carrier-bound DnsEtOGal are 3 and 20 ns, respectively (Table I). Using Eqn. 5, the apparent diffusion constants for the collisional encounter between fluorophore and quencher can be estimated. D is found to be decreased by a factor of 4 upon binding of DnsEtOGal to the carrier (Table I). As in the case of quenching of the MalNPyr-carrier by MePic, a reduction of D by a factor of 2 can be attributed to the immobilization of DnsEtOGal when bound to the carrier, so that the accessibility of the dansyl group is decreased by a factor of about 2 upon binding. The quenching of the fluorescence of carrier-bound DnsHxSGal by MePic (Table I) implies a similar reduction of the accessibility of the dansyl group upon binding of this galactoside to the carrier. This decrease cannot result from electrostatic repulsion between the positively charged MePic and the carrier, because using negatively charged NO_3^- as quencher essentially the same decrease in accessibility is found. Therefore, these observations suggest that the reduction in accessibility originates from steric effects and thus indicate that the dansyl moieties of DnsEtOGal and DnsHxSGal are not fully exposed to the aqueous phase at the water-protein interface.

Discussion

In an attempt to locate the galactoside binding site in the lactose carrier of *E. coli*, we have investigated the quenching of two types of fluorophores: MalNPyr attached to the carrier at the essential cysteine residue 148 and the substrates DnsEtOGal and DnsHxSGal. Briefly summarized, our findings are as follows. (1) Upon incorporation into the carrier the pyrenyl moiety of MalNPyr becomes less accessible to water-soluble quenchers. (2) The fluorophore is accessible to lipid-soluble quenchers and (3) is more accessible to these quenchers at the level of the C-5 of the lipid chains than to those at positions C-1 or C-12. (4) The dansyl moiety of specifically bound dansyl

galactosides resides in a hydrophobic environment, markedly hydrophobic for DnsEtOGal and slightly less hydrophobic for DnsHxSGal. (5) The dansyl groups are not accessible to lipid-soluble quenchers but (6) are accessible to a water-soluble quencher. We will now attempt to incorporate these observations into a rudimentary structural model of the lactose carrier.

The architectural principles for the folding of the lactose carrier must satisfy two somewhat disparate requirements. The protein is embedded in the membrane lipids and, hence, must present a hydrophobic surface towards the lipid phase. In contrast, the carrier must accept substrates, galactosides and H^+, from the aqueous phase and transport them through the matrix of the protein; hence, the carrier must present a partially hydrophilic surface along this pathway of the substrates. Experimentally, there is evidence that, at least for facilitated diffusion, the carrier functions as a monomer [24]. Thus, a single polypeptide chain must be folded in a way to create a partially hydrophilic pore shielded from the lipid phase.

Experimental studies and theoretical considerations suggest that the membrane-incorporated parts of proteins are preferentially in the α-helix conformation, which guarantees optimal formation of hydrogen bonds [27]. Therefore, as a first proposal for the architecture of a membrane protein a number of bilayer spanning α-helices may be placed side by side to form a closed ring in the plane of the membrane. On the sides facing the lipid phase, the helices must be hydrophobic, whereas the sides towards the interior of the ring may be partially hydrophilic, thus forming a partially hydrophilic pore. Such an architecture is reminiscent of the structure of the best-known membrane protein, bacteriorhodopsin, which probably consists of seven helices [28]. For the lactose carrier, a high α-helix content has been observed by circular dichroism measurements and 12 bilayer-spanning α-helices have been predicted on the basis of a hydrophobicity plot [5]. Not all these helices need be present in the ring at the protein-lipid interface. Some helices may reside solely in the somewhat more hydrophilic interior, surrounded by the outer ring of the more hydrophobic helices. In the interior nonhelical structures could occur as well. Thus the simplest model for the lactose permease would be a ring of bilayer-spanning α-helices possibly enclosing a partially hydrophilic interior with both α-helical and non-α-helical stretches of polypeptide chain folding.

Our results can easily be incorporated into this model, leading to a further refinement. Because the pyrenyl group at Cys-148 is accessible from the lipid phase, Cys-148 may be located on the inner surface of one of the helices of the ring. The pyrenyl moiety could project from Cys-148 between two helices into the lipid phase. Inspection of space-filling models discloses that there is little rotational freedom in Cys-MalNPyr, where Cys is located in an α-helix, due to steric hindrance. Moreover, investigations of the carrier-bound pyrenyl group by fluorescence anisotropy indicate that the fluorophore is relatively immobilized (L. Best and K. Dornmair, unpublished data). The simplest structural arrangement accommodating these various considerations locates the pyrenyl moiety and the Cys-148 residue at the same depth in the membrane; viz., Cys-148 is also located near the C-5 position of the lipid chains (Fig. 10). Finally, positing a partial overlap between Cys-148 and the binding site, the binding site is positioned within the carrier, not necessarily at the C-5 position, but between the faces of the bilayer and not at the membrane surface.

This result complements the fluorescence characterization of specifically bound dansyl galacto-

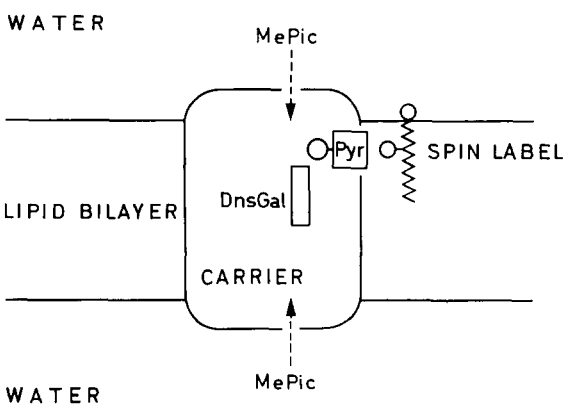

Fig. 10. Schematic representation of the lactose:H^+ carrier summarizing the experimental findings. The dansyl galactoside may equally well be shifted vertically to approach the cysteinyl-MalNPyr moiety.

sides which indicate a strongly hydrophobic environment of the dansyl groups, corresponding to a dielectric constant of 2–4. Such low values of the dielectric constant are incompatible with a position of the dansyl groups at the carrier-water interface, but rather require a position inside the carrier. On the other hand, the dansyl groups are accessible from the aqueous phase and not accessible from the lipid phase. This leads to the conclusion that the rod-shaped dansyl galactosides are located in a pore formed by the carrier, with the dansyl groups in a lateral environment of hydrophobic amino acid residues and accessible from the aqueous phase through the pore. The contact with water is established in the simplest way if the dansyl galactosides are oriented with the galactoside in the middle of the membrane and the dansyl group objected towards the aqueous phase. This would imply that the galactoside binding site is located rather deep in the pore, even deeper than the essential cysteine residue 148, which was found to be near the C-5 position of the lipid chains. Such detailed statements border on speculation, however.

In summary, we have shown that the binding site of the lactose carrier is located inside the protein in a two-fold sense: inside with respect to the lipid phase and inside with respect to the aqueous phase. The latter result is in accordance with the conclusion of Goldkorn et al. [29] drawn from topological studies of the lactose carrier using proteinases. The binding site is, however, accessible from the aqueous phase as expected for a protein transporting galactosides across the membrane.

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